

**This Page Is Inserted by IFW Operations  
and is not a part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



102

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 47/48</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 98/40104</b> <b>(43) International Publication Date:</b> 17 September 1998 (17.09.98)
<b>(21) International Application Number:</b> PCT/US98/04852 <b>(22) International Filing Date:</b> 11 March 1998 (11.03.98)  <b>(30) Priority Data:</b> 08/815,890      12 March 1997 (12.03.97)      US  <b>(71) Applicants:</b> YALE UNIVERSITY [US/US]; Room 210, 155 Whitney Avenue, P.O. Box 208336, New Haven, CT 06520-8336 (US). THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 308 Bynum Hall, CB #4105, Chapel Hill, NC 27599-4105 (US).  <b>(72) Inventors:</b> CHENG, Yung-Chi; 961 Baldwin Road, Woodbridge, CT 06525 (US). GUO, Xin; 7693 Palmilla Drive #2309, San Diego, CA 92122 (US). LEE, Kuo-Hsiung; 1426 Gray Bluff Trail, Chapel Hill, NC 27514 (US). BAS-TOW, Kenneth, F.; 1968 Lystra Road, Chapel Hill, NC 27514-8947 (US). WANG, Hui-Kang; 105 Fidelity Street, Carrboro, NC 27510-2062 (US).  <b>(74) Agents:</b> MAGRI, Karen, A. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US).		<b>(81) Designated States:</b> AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> COVALENT CONJUGATES OF TOPOISOMERASE I AND TOPOISOMERASE II INHIBITORS  <b>(57) Abstract</b>  The present invention provides covalent conjugates of topoisomerase I and topoisomerase II inhibitors. Such compounds have a structure according to the formula (I): $T_I-L-T_{II}$ wherein: $T_I$ is a topoisomerase I inhibitor such as a camptothecin group; $T_{II}$ is a topoisomerase II inhibitor such as an amsacrine, ellipticine, epipodophyllotoxin, or anthracycline antibiotic group; and L is a linking group. The compounds are useful for inhibiting topoisomerase I and II enzymes, for promoting cellular differentiation, and for treating cancer.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## COVALENT CONJUGATES OF TOPOISOMERASE I AND TOPOISOMERASE II INHIBITORS

This Invention was made with Government support under Grant No. CA 17625 from the National Cancer Institute. The Government has certain rights to this invention.

### Field of the Invention

5           The present invention concerns covalent conjugates of topoisomerase I inhibitors (*e.g.*, camptothecins) and topoisomerase II inhibitors (*e.g.*, amsacrine, ellipticines, anthracycline antibiotics, epipodophyllotoxins).

### Background of the Invention

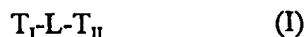
10           Etoposide is a widely-used antineoplastic agent which inhibits mammalian DNA topoisomerase II isoenzymes. *See*, F. Drake et al., *Biochem.* 28:8154 (1989); P. Watt and I. Hickson, *Biochem. J.* 303:681 (1994); and Y. Pommier, *Cancer Chemo. & Pharmac.* 32:103 (1993). Various etoposide derivatives have been developed in order to improve antitumor activity, cytotoxicity against drug resistant cells and drug-formulation characteristics  
15           including the 4'-O-demethylepipodophyllotoxins bearing C-4 $\beta$ -N-linked substituents. *See*, Y. Zhang and K. H. Lee, *Chin. Pharm. J.* 46:319 (1994). The other mammalian DNA topoisomerase, a type I enzyme, is also considered to be a useful therapeutic target. Several selective inhibitors have been identified to date including the antitumor alkaloid, camptothecin, (CPT)

and two analogs are currently approved for clinical use in the United States. See, P. Watt and I. Hickson, *Biochem. J.* 303:681 (1994); and A. Chen and L. Liu, *Annu. Rev. Pharmacol. Toxicol.* 34:191 (1994). However, not all topoisomerase inhibitors of potential clinical value are topoisomerase-type specific. For example, a 7-H-benzopyrido (4,3-b) indole-derivative (inotoplicine), inhibits topoisomerases I and II simultaneously and can circumvent topoisomerase-mediated mechanisms of drug-resistance. See, B. Podderin et al., *Mol. Pharmacol.* 44:767 (1993).

All of the above-mentioned compounds share a common inhibitory mechanism which is understood in some depth at the biochemical level. Enzyme inhibition involves the trapping of a putative covalent reaction intermediate called a reversible "cleavable complex". The intracellular lesion, presumably a topoisomerase-DNA-drug ternary complex, ultimately leads to cell death. Although the cytotoxic events depend on the particular type of topoisomerase involved, the precise biochemical pathway(s) to cell killing remains to be defined. See, P. Watt and I. Hickson, *Biochem. J.* 303:681 (1994); A. Chen and L. Liu, *Annu. Rev. Pharmacol. Toxicol.* 34:191 (1994); and P. Darpa et al., *Cancer Res.* 50:6919 (1990).

#### Summary of the Invention

A first aspect of the present invention is covalent conjugates of topoisomerase I and topoisomerase II inhibitors. Such compounds have a structure according to formula I:



wherein:

- $T_I$  is a topoisomerase I inhibitor such as a camptothecin group;  
 $T_{II}$  is a topoisomerase II inhibitor such as an amsacrine, ellipticine, epipodophyllotoxin, anthracycline antibiotic group, or mitoxantrone group; and  
L is a linking group.

The compounds of formula I are useful as, among other things, inhibitors of topoisomerase I and topoisomerase II.

The present invention is explained in greater detail in the drawings herein and the specification set forth below.

#### Brief Description of the Drawings

5  
10  
15  
Figures 1A and 1B. Topoisomerase-DNA complex formation *in vitro*. A. Potassium-SDS precipitable radioactivity recovered from cleavage reactions containing topoisomerase I (stippled boxes) and topoisomerase II (shaded boxes), alone or with compounds 1-6 at ten micromolar. Input radioactivity was about 7000 P<sup>32</sup> cpm. Data are single determinations from the same experiment. B. Agarose gel autoradiogram of topoisomerase II-induced DNA cleavage products. Treatment conditions are indicated above the lanes and potassium-SDS precipitable radioactivity recovered are given below. Input radioactivity was about 5000 P<sup>32</sup> cpm. DNA fragments discussed in the text are indicated by small open circles in the right margin of lane 7. Non-adjacent lanes from a single gel autoradiogram were juxtaposed to prepare this figure.

20  
Figures 2A and 2B. Protein-associated DNA breaks in KB cells. Treatments were for one hour with test concentrations indicated on the ordinate. Etoposide C-1 ( $\Delta$ ); Compounds 3 ( $\square$ ), 4b ( $\blacksquare$ ), S-2 ( $\bullet$ ) and co-treatments with 3 and 4b (dotted line). Data obtained from triplicate treatments in a single experiment. Bars, SD. The effect of treatments at low concentration (0.5-2.5  $\mu$ M) in 3A are results from 3B replotted for clarity.

25  
30  
Figure 3. Reversible genomic DNA fragmentation detected by a gel lysis assay method. KB cells were immobilized in agarose plugs, lysed and analyzed as described in "Material and Methods". Cells were prepared immediately following three hours of treatment (lanes 3, 5, 7, 8, 10 and 11) or following an additional hour of incubation in fresh growth medium lanes 4, 6, 9 and 12). Treatments are indicated under the sample lanes and concentrations used are given in the text under "Results". The first two lanes are control samples from KB cells mock-treated with 0.5% (v/v) dimethylsulfoxide as a

carrier control; lane 1 is DNA from cells treated for three hours and the other sample was prepared following a one hour treatment reversal. Arrows in the left margin indicate the plug position at the gel origin and the mobility of phage lambda DNA molecular mass markers in Kb.

5                   **Figures 4A and 4B.** *In vivo* effects of compounds S-1 and S-2 in nude mice with KB cell tumor development. In **Figure 4A**: control (—●—), etoposide C-1 (---Δ---), camptothecin C-2 (--○--), S-1 at 3 mg/kg/day (--■--), S-1 at 7.5 mg/kg/day (--▲--), and S-1 at 15 mg/kg/day (-·▼-·). In **Figure 4B**: control (—●—), etoposide C-1 (---Δ---), camptothecin C-2 (--○--), S-2 at 15  
10 mg/kg/day (--▼--), and S-2 at 50 mg/kg/day (--○--).

#### Detailed Description of the Invention

As used herein the term "alkyl" refers to C<sub>1-20</sub> inclusive, linear, branched, or cyclic, saturated or unsaturated hydrocarbon chains, including for example, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, pentyl,  
15 hexyl, octyl, ethenyl, propenyl, butenyl, pentenyl, hexenyl, octenyl, butadienyl, and allenyl groups. Thus, the term "alkyl" refers to C<sub>1-20</sub> inclusive alkyls unless otherwise specified. The term "alkylene" refers to C<sub>1-20</sub> inclusive alkyls. The term "alkenyl" refers to C<sub>1-20</sub> inclusive alkyls containing one or more double bonds. The term "alkoxy" as used herein refers to C<sub>1-20</sub> inclusive,  
20 linear, branched, or cyclic, saturated or unsaturated oxo-hydrocarbon chains, including for example methoxy, ethoxy, propoxy, isopropoxy, butoxy, t-butoxy, and pentoxy. The term "aryloxy" as used herein refers to phenyloxy and alkyl or alkoxy substituted phenyloxy. The term "arylene" as used herein refers to phenylene and alkyl or alkoxy substituted phenylene. The  
25 terms "halo," "halide," or "halogen" as used herein refer to fluorine, chlorine, bromine, and iodine.

As noted above, the present invention is concerned with compounds that are active as topoisomerase I and topoisomerase II inhibitors. Such compounds have a structure according to formula I:

30

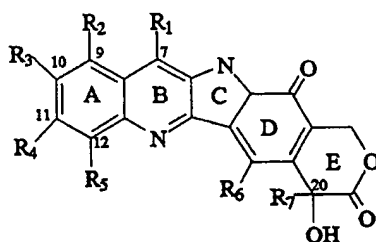
T<sub>I</sub>-L-T<sub>II</sub>

(I)

wherein  $T_I$  is a topoisomerase I inhibitor,  $T_{II}$  is a topoisomerase II inhibitor, and L is a covalent linking group. As will be appreciated by those skilled in the art, the topoisomerase I inhibitor may be either a selective topoisomerase I inhibitor (that is, have essentially no topoisomerase II inhibitory activity; for example, a camptothecin group) or may be a mixed inhibitor (that is, have both topoisomerase I and topoisomerase II inhibitory activity). Likewise, the topoisomerase II inhibitor may be either a selective topoisomerase II inhibitor (that is, have essentially no topoisomerase I inhibitory activity; for example, an amsacrine, ellipticine, epipodophyllotoxin, anthracycline antibiotic group or mitoxantrone) or may be a mixed inhibitor. Preferably, at least one of the topoisomerase I and topoisomerase II inhibitors is a selective inhibitor, and more preferably both of the topoisomerase I and topoisomerase II inhibitors are selective inhibitors.

#### A. TOPOISOMERASE I INHIBITORS.

Particularly preferred topoisomerase I inhibitors are camptothecin groups (the term "camptothecin group" is intended to encompass camptothecin and analogs thereof). Such compounds are typically of the formula II:



II

wherein:

$R_1$  is selected from the group consisting of H, alkyl, alkoxy, alkylaryl, hydroxyalkyl, haloalkyl, aminoalkyl, dialkylamino, dialkylaminoalkyl, cycloaminoalkyl, aryl, aryloxy, C-glycal,  $CO_2R_7$ , nitro, cyano, halo,  $SR_8$ ,  $NR_8R_8$ , O-glycosyl and  $L-T_{II}$ ; and

$R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are each independently H, amino, hydroxy, alkyl, alkoxy, alkylthiol, alkylamino, aminoalkyl, di(alkyl)amino, cycloaminoalkyl, aminoalkoxy, aryl, aryloxy, C-glycal, cyano, methylenedioxy,



formyl, nitro, halo, trifluoromethyl, aminomethyl, azido, amido, hydrazino, any of the twenty standard amino acids bonded to the A ring via the amino-nitrogen atom,  $SR_8$ ,  $NR_8R_8$ , or O-glycosyl,  $L-T_{II}$ ; or  $R_3$  and  $R_4$  together form a 5- or 6-member aromatic or dioxolane ring; and wherein methylenedioxy  
5 comprises  $R_2$  and  $R_3$ ,  $R_3$  and  $R_4$ , or  $R_4$  and  $R_5$  taken together;

$R_7$  is ethyl; and

each  $R_8$  is independently selected from the group consisting of H, alkyl, alkylaryl, hydroxyalkyl, aminoalkyl, acyl, or aryl;

subject to the proviso that one of  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  is a  
10 substituent of the formula  $-L-T_{II}$ , wherein

L is a covalent linking group; and

$T_{II}$  is a topoisomerase II inhibitor.

Substituents on the "A" ring of the compounds of formula II may be joined together to form a bifunctional substituent such as the methylenedioxy  
15 group. Methylenedioxy substituents may be bonded to any two consecutive positions in the A ring, for example, the 9,10, the 10,11, or the 11,12 positions.

Substituents which are standard amino acids may be any of the twenty amino acids commonly found in naturally occurring proteins, and are  
20 well known in the art. These provide a substituent of the formula  $-NHCHR_1COOH$ , with R being the side chain of any of the twenty standard amino acids. The amino acids may be of any configuration, but preferably have an (L) configuration.

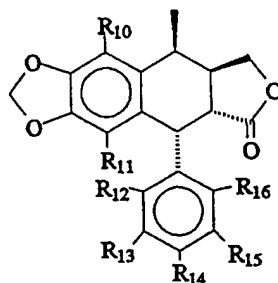
## B. TOPOISOMERASE II INHIBITORS.

25  $T_{II}$  is a topoisomerase II inhibitor. Suitable topoisomerase II inhibitor groups include groups which inhibit both topoisomerase I and topoisomerase II as well as groups which inhibit topoisomerase II selectively. As will be apparent to those skilled in the art, the conjugates of the present invention are comprised of one group, which alone is a topoisomerase I  
30 inhibitor, and one group, which alone is a topoisomerase II inhibitor, and a linking group which joins the foregoing groups to form the conjugate.

Accordingly by the statement that the  $T_{II}$  group is a topoisomerase II inhibitor is meant that  $T_{II}$  is a group derived from and based upon a parent compound which exhibits topoisomerase II inhibition activity. Accordingly, there are a number of topoisomerase II inhibitor compounds from which the topoisomerase II inhibiting group  $T_{II}$  may be drawn, which compounds are well known to those skilled in the art. For example, suitable topoisomerase II inhibiting compounds are those compounds within the general classes of epipodophyllotoxins, amsacrine, ellipticines, anthracycline antibiotics, and mitoxantrones.

### 1. Epipodophyllotoxin groups.

One suitable class of compounds within the scope of the definition of  $T_{II}$  is the epipodophyllotoxin class of compounds. Suitable topoisomerase II inhibiting groups derived from the epipodophyllotoxin class of compounds include groups of the general formula III-A:



III-A

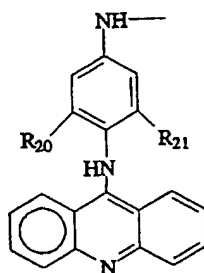
wherein  $R_{10}$ ,  $R_{11}$ ,  $R_{12}$ , and  $R_{16}$  are each independently selected from the group consisting of H and halo;  $R_{13}$  and  $R_{15}$  are each independently alkoxy; and  $R_{14}$  is selected from the group consisting of H, hydroxyl, alkyl, and phosphate salt. As will be apparent to the skilled artisan, the unoccupied bond represents the location at which this particular  $T_{II}$  group is joined to the linking group L.

Specific examples of preferred  $T_{II}$  groups of formula III-A include groups wherein  $R_{10}$ ,  $R_{11}$ ,  $R_{12}$ , and  $R_{16}$  are each H; groups wherein  $R_{13}$  and  $R_{15}$  are each methoxy; and groups wherein  $R_{14}$  is hydroxyl.

Specific examples of topoisomerase II inhibiting compounds from which the  $T_{II}$  group may be derived include but are not limited to etoposide and teniposide.

## 2. Amsacrine groups.

5 Another suitable class of compounds within the scope of the definition of  $T_{II}$  is the amsacrine class of compounds. Suitable topoisomerase II inhibiting groups derived from the amsacrine class of compounds include groups of the general formula III-B:



III-B

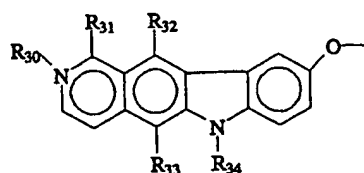
10 wherein  $R_{20}$  and  $R_{21}$  are independently selected from the group consisting of alkoxy and alkylhydroxyl. As will be apparent to the skilled artisan, the unoccupied bond represents the location at which this particular  $T_{II}$  group is joined to the linking group L.

Specific examples of preferred  $T_{II}$  groups of formula III-B  
15 include those compounds wherein  $R_{20}$  is methoxy; and groups wherein  $R_{21}$  is  $-CH_2OH$ .

Specific examples of topoisomerase II inhibiting compounds from which the  $T_{II}$  group may be derived include but are not limited to *m*-AMSA.

## 3. Ellipticine groups.

20 Another suitable class of compounds within the scope of the definition of  $T_{II}$  is the ellipticine class of compounds. Suitable topoisomerase II inhibiting groups derived from the ellipticine class of compounds include groups of the general formula III-C:



III-C

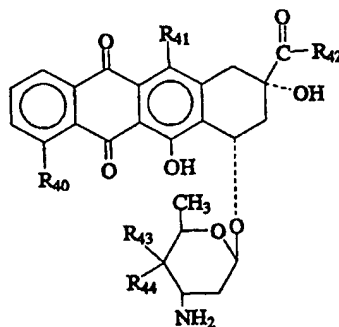
wherein  $R_{30}$  is H or aliphatic amine;  $R_{31}$  is H or  
 $-C(O)-N(R_{35})-(CH_2)_n-N(R_{36})(R_{37})$  wherein  $R_{35}$ ,  $R_{36}$ , and  $R_{37}$  are each  
independently selected from the group consisting of H and alkyl and  $n$  is an  
5 integer from 1 to 6;  $R_{32}$  and  $R_{34}$  are each independently selected from the group  
consisting of H and alkyl; and  $R_{33}$  is alkyl. As will be apparent to the skilled  
artisan, the unoccupied bond represents the location at which this particular  $T_{II}$   
group is joined to the linking group L.

Specific examples of preferred  $T_{II}$  groups of formula III-C  
10 include those groups wherein  $R_{30}$  is H; groups wherein  $R_{30}$  is an aliphatic amine  
particularly, propylamine and N-diethylpropylamine; groups wherein  $R_{31}$  is H;  
groups wherein  $R_{32}$  is alkyl, particularly methyl; groups wherein  $R_{33}$  is methyl;  
and groups wherein  $R_{34}$  is H.

Specific examples of topoisomerase II inhibiting compounds from  
15 which the  $T_{II}$  group may be derived include but are not limited to ellipticine.

#### 4. Anthracycline antibiotic groups.

Another suitable class of compounds within the scope of the  
definition of  $T_{II}$  is the anthracycline antibiotic class of compounds. Suitable  
topoisomerase II inhibiting groups derived from the anthracycline antibiotic  
20 class of compounds include groups of the general formula III-D:



III-D

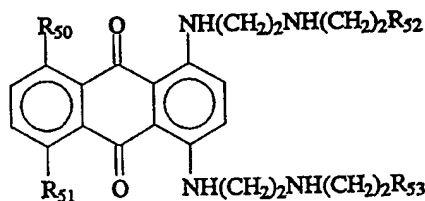
wherein  $R_{40}$  is H or alkoxy;  $R_{41}$  is hydroxyl or O—;  $R_{42}$  is alkylhydroxyl, or  $CH_2O$ —; and  $R_{43}$  and  $R_{44}$  are each independently selected from the group consisting of H and hydroxyl; subject to the provisos that if  $R_{41}$  is hydroxyl, then  $R_{42}$  is  $CH_2O$ —; and if  $R_{42}$  is alkyl or alkylhydroxyl, then  $R_{41}$  is O—. As  
 5 is apparent to those skilled in the art, the unoccupied bond (i.e., O— or  $CH_2O$ —) represents the location at which this particular  $T_{II}$  group is joined to the linking group L.

Specific examples of preferred  $T_{II}$  groups of formula III-D include those groups wherein  $R_{40}$  is alkoxy, particularly methoxy; groups  
 10 wherein  $R_{41}$  is hydroxyl; groups wherein  $R_{41}$  is O—; groups wherein  $R_{42}$  is alkylhydroxyl; groups wherein  $R_{42}$  is  $CH_2O$ —; groups wherein  $R_{43}$  is H and  $R_{44}$  is hydroxyl. As will be clear to those skilled in the art, at least one of  $R_{41}$  and  $R_{42}$  must possess the bond joining the  $T_{II}$  group to L. Hence, if  $R_{41}$  is not O—, then  $R_{42}$  must be  $CH_2O$ —.  $R_{41}$  must not be O— if  $R_{42}$  is  $CH_2O$ —.

15 Specific examples of topoisomerase II inhibiting compounds from which the  $T_{II}$  group may be derived include but are not limited to adriamycin, doxorubicin, daunorubicin, epirubicin, and idarubicin.

### 5. Mitoxantrone groups.

Another suitable class of compounds within the scope of the  
 20 definition of  $T_{II}$  is the mitoxantrone class of compounds. Suitable topoisomerase II inhibiting groups derived from the mitoxantrone class of compounds include groups of the general formula III-E:



III-E

wherein  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  are each independently selected from the group  
 25 consisting of OH or O—; subject to the proviso that one of  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  is O—. As is apparent to those skilled in the art, the unoccupied bond

(i.e., O—) represents the location at which this particular  $T_{II}$  group is joined to the linking group L.

Specific examples of preferred  $T_{II}$  groups of formula III-E include those groups wherein  $R_{S2}$  is O—; and compounds wherein  $R_{S0}$  is O—.

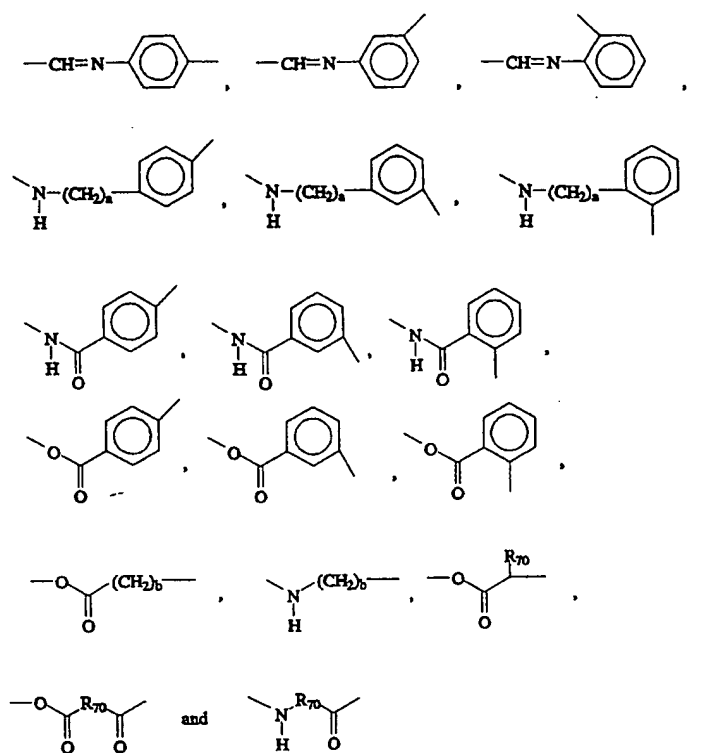
5           Specific examples of topoisomerase II inhibiting compounds from which the  $T_{II}$  group may be derived include but are not limited to mitoxantrone.

### C. LINKING GROUPS.

The specific linking group employed will depend upon the particular synthetic method used to make the covalent conjugate, as will be appreciated by those skilled in the art. The linking group "L" may be  
10           considered as a separate group, as in formula I above, or may be considered as a side-chain of either (or both) of the topoisomerase I and the topoisomerase II inhibitor.

Many suitable linking groups will be determinable by those skilled in the art. A suitable linking group will permit the joining of the  
15           topoisomerase I (i.e.,  $T_I$ ) and topoisomerase II ( $T_{II}$ ) inhibitors to provide a metabolically stable conjugate (i.e., a conjugate for which steric hinderance will not be so strong as to prevent the joining of the  $T_I$  and  $T_{II}$  groups).

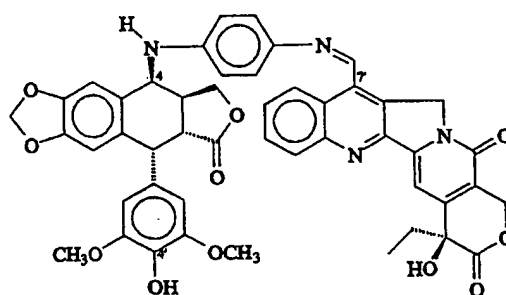
Specific examples of suitable linking groups within the scope of  
20           the definition of L include but are not limited to:



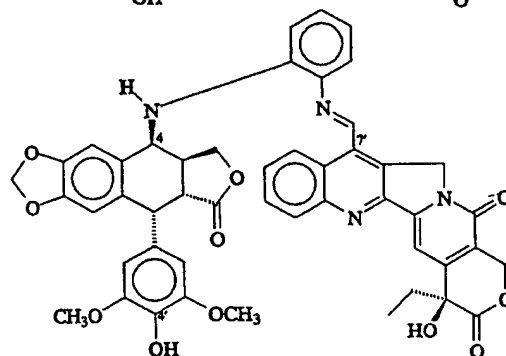
wherein a is a 0-3; b is a 0-3; and  $\text{R}_{70}$  is selected from the group consisting of alkylene, alkenyl, and arylene.

#### D. EXAMPLE COMPOUNDS.

Specific examples of compounds within the scope of the present invention include but are not limited to:



S-1



S-2

### E. SYNTHESIS OF COMPOUNDS.

A wide variety of the topoisomerase I and topoisomerase II  
5 inhibiting conjugates of the present invention can be prepared according to the  
following general techniques. Variations on the following general synthetic  
method will be readily apparent to those skilled in the art and are deemed to be  
within the scope of the present invention. In general, the conjugates of formula  
I may be prepared by refluxing a topoisomerase I inhibitor compound with a  
10 topoisomerase II inhibitor compound in tetrahydrofuran.

As a specific example, the conjugates S-1 and S-2 above may be  
prepared by dissolving 7-formyl camptothecin and 4-demethylepipodophyllo-  
toxin derivatives in dry tetrahydrofuran. Then 1.0 M hydrogen chloride ether  
solution is added to adjust the pH to 1-2. The solution is refluxed for 1 hour  
15 and evaporated to dryness. The reaction produces the compounds in 70-80%  
yield. As will be apparent to those skilled in the art, the structures of the



conjugates produced may be determined using conventional spectroscopic and analytical techniques.

#### F. PHARMACEUTICAL FORMULATIONS.

5 The topoisomerase I and topoisomerase II inhibiting compounds of formula I of the present invention are useful as pharmaceutically active agents and may be utilized in bulk form. More preferably, however, these compounds are formulated into pharmaceutical formulations for administration. Any of a number of suitable pharmaceutical formulations may be utilized as a vehicle for the administration of the compounds of the present invention.

10 The compounds of Formula I may be formulated for administration for the treatment of a variety of conditions. In the manufacture of a pharmaceutical formulation according to the invention, the compounds of Formula I and the physiologically acceptable salts thereof, or the acid derivatives of either (hereinafter referred to as the "active compound") are typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a tablet, which may contain from 0.5% to 95% by weight of the active compound. One or more active compounds may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory ingredients.

25 The formulations of the invention include those suitable for oral, rectal, topical, buccal (e.g., sub-lingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous) and transdermal administration, although the most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular active compound which is being used.

30

Formulations suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Such formulations may be prepared by any suitable method of pharmacy which includes the step of bringing into association the active compound and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the formulations of the invention are prepared by uniformly and intimately admixing the active compound with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the active compound, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the compound in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

Formulations suitable for buccal (sub-lingual) administration include lozenges comprising the active compound in a flavoured base, usually sucrose and acacia or tragacanth; and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Formulations of the present invention suitable for parenteral administration conveniently comprise sterile aqueous preparations of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may be administered by means of subcutaneous, intravenous, intramuscular, or intradermal injection. Such preparations may conveniently be prepared by admixing the compound with water or a glycine buffer and rendering the resulting solution sterile and isotonic with the blood.

Formulations suitable for rectal administration are preferably presented as unit dose suppositories. These may be prepared by admixing the

active compound with one or more conventional solid carriers, for example, cocoa butter, and then shaping the resulting mixture.

Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include vaseline, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration may also be delivered by iontophoresis (see, for example, *Pharmaceutical Research* 3(6):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound. Suitable formulations comprise citrate or bis\tris buffer (pH 6) or ethanol/water and contain from 0.01 to 0.2M active ingredient.

#### G. UTILITIES AND METHODS OF USE.

In addition to the compounds of formula I, the present invention also provide useful therapeutic methods. For example, the present invention also provide a method of inhibiting topoisomerase I and topoisomerase II enzymes. The method includes contacting topoisomerase I and topoisomerase II *in vitro* or *in vivo* with an inhibitory effective amount of a compound of formula I. The inhibition of topoisomerase I and II is a useful means of inhibiting cancer cell growth.

The present invention also provides a method of inducing cellular differentiation. The method includes contacting a cancer cell with a differentiation effective amount of a compound of formula I. Cancer cells which may be differentiated include cells from small cell lung cancer, testicular cancer, lymphoma, leukemia, esophageal cancer, stomach cancer, colon cancer, breast cancer, central nervous system cancer, liver cancer and prostate cancer.

The present invention also provides a method of treating cancer in a subject afflicted with cancer. The method includes administering to the

subject in an effective cancer treating amount a compound of formula I. The methods are useful for the treatment of a variety of cancer cells which include but are not limited to small cell lung cancer, testicular cancer, lymphoma, leukemia, esophageal cancer, stomach cancer, colon cancer, breast cancer, central nervous system cancer, liver cancer and prostate cancer.

Subjects which may be treated using the methods of the present invention are typically human subjects although the methods of the present invention may be useful for veterinary purposes with other subjects, particularly mammalian subjects including, but not limited to, horses, cows, dogs, rabbits, fowl, sheep, and the like. As noted above, the present invention provides pharmaceutical formulations comprising the compounds of Formula I, or pharmaceutically acceptable salts thereof, in pharmaceutically acceptable carriers for any suitable route of administration, including but not limited to oral, rectal, topical, buccal, parenteral, intramuscular, intradermal, intravenous, and transdermal administration.

The therapeutically effective dosage of any specific compound will vary somewhat from compound to compound, patient to patient, and will depend upon the condition of the patient and the route of delivery. As a general proposition, a dosage from about 0.1 to about 50 mg/kg will have therapeutic efficacy, with still higher dosages potentially being employed for oral and/or aerosol administration. Toxicity concerns at the higher level may restrict intravenous dosages to a lower level such as up to about 10 mg/kg, all weights being calculated based upon the weight of the active base, including the cases where a salt is employed. Typically a dosage from about 0.5 mg/kg to about 5 mg/kg will be employed for intravenous or intramuscular administration. A dosage from about 10 mg/kg to about 50 mg/kg may be employed for oral administration.

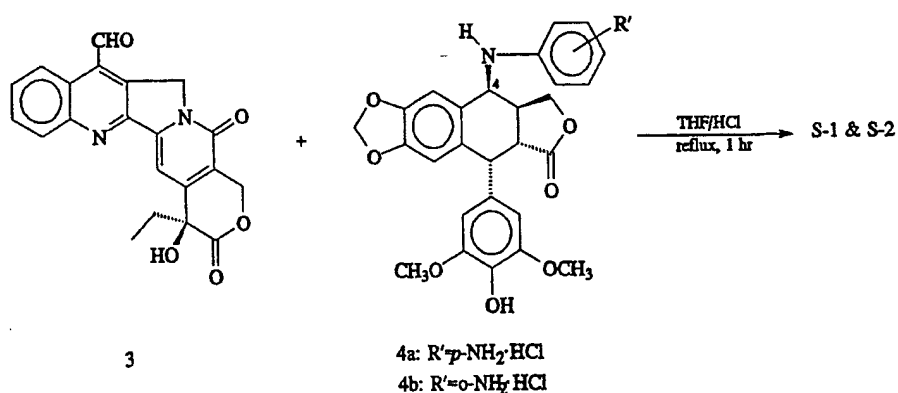
The present invention is explained in greater detail in the following non-limiting examples, wherein the following abbreviations are used: "THF" means tetrahydrofuran; "SDS" means sodium dodecylsulfate; "PBS" means 140mM NaCl, 4mM KCl, 0.5mM Na<sub>2</sub>HPO<sub>4</sub>, 150mM KH<sub>2</sub>PO<sub>4</sub>; "TBE" means 95mM tris-borate, 2mM EDTA pH 8; "mAMSA" means 4'-(9-

acridinylamino)-methanesulfon-m-anisidide; "EDTA" means disodium(ethylenedinitrilo) tetraacetic acid; "RN'ase" means bovine pancreatic ribonuclease; and "PFGE" means pulsed-field gel electrophoresis.

### EXAMPLE 1

#### Conjugate Synthesis

Compounds S-1 and S-2 were synthesized according to the procedure shown in the following Scheme 1:



In general, 7-formyl camptothecin (1 eq.) and 4-demethylepipodophyllotoxin derivatives (4a or 4b, 1 eq.) were dissolved in dry THF, and 1.0 M hydrogen chloride ether solution was added to adjust the pH to 1-2. The solution was refluxed for 1 hour and evaporated to dryness. The residue was purified via column chromatography (silica gel with chloroform methanol, 100:1 (v/v) as the eluent) to give target compounds in 70-80% yield. The structures of final products were secured by spectroscopic and analytical data.

Compound S-1, red crystals, mp >300°C (decomp.), IR (KBr) cm<sup>-1</sup> 3350 (broad, OH, NH), 1770 (sh., δ-lactone), 1650 (carbonyl of amide); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 1.06 (t, J = 7.5 Hz, 3H, 18'''-H), 1.97 (q, J = 7.5 Hz, 2H, 19'''-H), 3.14 (m, 1H, 3-H), 3.28 (dd, J = 4.9, 14.0 Hz, 1H, 2-H), 3.80 (s, 6H, OCH<sub>3</sub> x 2), 4.05, 4.48 (t, J = 8.7 Hz, 1H each, 11-H), 4.62 (d, J = 4.9 Hz, 1H, 1-H), 4.86 (d, J = 3.9 Hz, 1H, 4-H), 5.35, 5.69 (ABq, J = 16.0

Hz, 1H each, 17'''-H), 5.72 (s, 2H, 5'''-H), 5.97 (d,  $J = 5.1$  Hz, 2H, OCH<sub>2</sub>O), 6.36 (s, 2H, 2', 6'-H), 6.54 (s, 1H, 8-H), 6.76 (d,  $J = 8.7$  Hz, 2H, 2'', 6''-H), 6.85 (s, 1H, 5-H), 7.56 (d,  $J = 8.7$  Hz, 2H, 3'', 5''-H), 7.76 (s, 1H, 13'''-H), 7.79, 7.89 (t,  $J = 8.0$  Hz, 1H each, 10''', 11'''-H), 8.25, 8.61  
 5 (d,  $J = 8.0$  Hz, 1H each, 9''', 12'''-H), 9.60 (s, 1H, N=CH); Anal. Calcd. for C<sub>48</sub>H<sub>40</sub>N<sub>4</sub>O<sub>11</sub> • H<sub>2</sub>O: C 66.50, H 4.88, N 6.46; Found C 66.81, H 4.90, N 6.50.

Compound S-2, red crystals, mp 255-256°C (decomp.), IR (KBr) cm<sup>-1</sup> 3350 (broad, OH, NH), 1770 (sh.,  $\delta$ -lactone), 1650 (carbonyl of amide);  
 10 <sup>1</sup>H NMR (CDCl<sub>3</sub>)  $\delta$ : 1.05 (t,  $J = 7.5$  Hz, 3H, 18'''-H), 1.91 (m, 2H, 19'''-H), 3.10 (m, 1H, 3-H), 3.37 (dd,  $J = 4.9, 14.2$  Hz, 1H, 2-H), 3.81 (s, 6H, OCH<sub>3</sub> x 2), 4.08 (dd,  $J = 2.9, 8.0$  Hz, 1H, 11-H), 4.46 (t,  $J = 8.0$  Hz, 1H, 11-H), 4.74 (d,  $J = 4.9$  Hz, 1H, 1-H), 4.84 (t-like, 1H, 4-H), 5.28 (s, 1H, 2''-H), 5.23, 5.42 (ABq,  $J = 20.5$  Hz, 1H each, 17'''-H), 5.30, 5.74 (ABq,  $J = 16.6$  Hz, 1H each, 5'''-H), 5.96 (d,  $J = 5.5$  Hz, 2H, OCH<sub>2</sub>O), 6.37 (s, 2H, 2', 6'-H), 6.49 (s, 1H, 8-H), 6.71, 6.90 (both d,  $J = 7.5$  Hz, 1H each, 2'', 5''-H), 6.86 (s, 1H, 5-H), 7.33, 7.35 (t,  $J = 7.5$  Hz, 1H, 3'', 4''-H), 7.62 (s, 1H, 13'''-H), 7.71, 7.87 (t,  $J = 8.0$  Hz, 1H each, 10''', 11'''-H), 8.28, 8.57 (d,  $J = 8.0$  Hz, 1H each, 9''', 12'''-H), 9.55 (s, 1H, N=CH);  
 15 Calcd. for C<sub>48</sub>H<sub>40</sub>N<sub>4</sub>O<sub>11</sub> • 0.5H<sub>2</sub>O: C 67.18, H 4.82, N 6.53; Found C 67.04, H 4.88, N 6.41.

## EXAMPLE 2

### Enzymes and Reagents

Calf thymus DNA topoisomerases were purchased from TopoGen  
 25 Inc., Columbus, Ohio. Tritium-labeled thymidine (60-90 ci/mmol) for labeling cellular DNA to measure protein-DNA complexes and  $\alpha$ P<sup>32</sup>-labeled deoxycytidine triphosphate (>3000 ci/mmol) were obtained from ICN Biochemicals Inc., Irvine, California. The latter radiochemical was used with a T4-DNA polymerase polymerase-labeling system from BRL Inc., Gaithersburg,  
 30 Maryland, to prepare P<sup>32</sup>-plasmid DNA for *in vitro* cleavage assays. Tissue

culture reagents were purchased from Sigma Chemical Co., St. Louis, Missouri, and Gibco-BRL Inc. All other chemicals were reagent grade.

### EXAMPLE 3

#### Drugs

5                   Etoposide and camptothecin were obtained from the Natural Products Laboratory, UNC-Chapel Hill. The derivatives 3, 4a and 4b were prepared according to published methods described in H.K. Wang et al., *Bioorg. & Med. Chem.* 2:1397 (1994) and Z.Q. Wang et al., *J. Med. Chem.* 33:2660 (1990). All compounds were dissolved to 20 mM final concentration in  
10               dimethylsulfoxide and stored at minus 70°C. Immediately before use, compounds were diluted in water for biochemical studies or in culture medium under sterile conditions for cell-based assays.

### EXAMPLE 4

#### Cell Lines and Cultures

15                   The KB oral carcinoma cell line was provided by M. Fisher (Pharmacology, UNC- Chapel Hill). The isolation and properties of the etoposide-resistant sub-clone, KB-7d, and the camptothecin-resistant line, KB-CPT100, were reported in P. Ferguson et al., *Cancer Res.* 48:5956 (1988) and D. Beidler et al., *Cancer Res.* 56:345 (1996), and are discussed briefly  
20               hereinafter. The vincristine-resistant sub-clone, KB-VIN20c was isolated by selection in 20 nM vincristine and over-expresses the P-glycoprotein multidrug-resistant drug-efflux protein (unpublished results).

                  All cell types were propagated in RPMI-1640 medium supplemented with 5% (v/v) fetal calf serum and 100 µg/mL kanamycin at 37°  
25               in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Over the course of the experiments, doubling times of cells were 24 ± 4 hours.

                  Fifty thousand cells were plated in 25cm<sup>2</sup> flasks (Falcon, New Jersey, USA) and incubated overnight. The medium was supplemented with test compounds at various concentrations and cultures were incubated for 48-51  
30               hours (for about two cell doublings) before cells were trypsinized and then

counted using a hemacytometer. The number of treated cells which excluded 0.5% (v/v) trypan blue were expressed as a percentage of the growth measured in control cultures. Compounds were evaluated in at least two independent experiments and the combined results were analyzed as scatter plots with dose-responses being computer fitted by linear regression at the 95% confidence level using Graphpad Software., San Diego, California. The  $IC_{50}$  concentrations which inhibited cell growth by fifty percent relative to control were interpolated from graphed results.

#### EXAMPLE 5

10

##### Cytotoxicity Assay

$LC_{50}$  concentrations were determined by treating cells for three hours with different concentrations of compounds then replating cells to measure colony formation and plating efficiency as the percent of total cells forming colonies. Compounds were evaluated in three independent experiments. The plating efficiencies of untreated cells varied between experiments with values ranging from 14-31% for KB, 31-56% for KB-7d, 16-38% for KB-VIN20c and 21-36% for KB-CPT100. A concentration of compound that reduced plating efficiency by fifty percent relative to control, the  $LC_{50}$ , was determined graphically as stated in the preceding section.

20

#### EXAMPLE 6

##### Cellular Protein-DNA Complex Formation

Stimulation of intracellular protein associated DNA breaks was measured using a standard assay method described in Y. Kashiwada et al., *J. Pharm. Sci.* 82:487 (1993). Briefly, KB cells were labeled overnight with tritiated thymidine (0.5  $\mu$ Ci/mL), chased for two hours and then treated in triplicate with test compounds at various concentrations. After one hour, samples were processed and protein-DNA complexes were measured as potassium-SDS precipitable radioactivity.



### EXAMPLE 7

#### In Vitro Topoisomerase-DNA Cleavage Assay

The detailed method used to measure stimulation of cleavable complexes *in vitro* is described in K. Bastow et al., *Planta Med.* 59:195 (1993).  
5 Briefly, incubated cleavage reactions containing one nanogram of P<sup>32</sup> end-labeled linearized PBR322 DNA, one unit of enzyme and test compound were divided equally for analysis. One portion was prepared for gel electrophoresis with subsequent autoradiography and protein-DNA complexes in the remaining sample were analyzed using the potassium-SDS precipitation method and  
10 scintillation spectrometry.

### EXAMPLE 8

#### Gel Lysis Assay Method for DNA Breaks

The procedure used to detect inhibitor-induced cleavable-complexes as genomic DNA fragmentation on agarose gels is based on the  
15 procedure described in P. Walker et al., *Biotechniques* 15:1032 (1993), with suggested modifications (Goz B, LaBiche R; personal communication) as follows. One million KB cells in 25cm<sup>2</sup> flasks were treated for three hours with test compound, the medium was discarded then cells were scraped into ice-cold PBS and harvested by centrifugation. Washed cell pellets were  
20 resuspended in eighty microliters of a 37° gel solution [1% (w/v) low-melting point agarose in PBS], and were cast in a mold and refrigerated to form 6 x 7 x 2 mm agarose plugs. Plugs of cells were incubated in lysis-digestion buffer (0.4M EDTA, 0.01 Tris (HCl, pH 8.0), 1% (w/v) N-lauryl-sarcosine and 100 µg/mL proteinase K) at 37° overnight or 50° for one hour. The treated plugs  
25 were equilibrated in TBE buffer and placed against a gel comb in a horizontal gel casting tray (BRL Inc., H4 system format). A 55° gel solution containing 0.8% (w/v) agarose in TBE was poured and allowed to gel. Electrophoresis was at 3.5 v/cm (measured between electrodes) for 16 hours at 4° without buffer re-circulation. The gel was stained with 1 µg/mL ethidium bromide in  
30 water, treated overnight at 25°C with RN'ase (1 µg/mL), and then photographed under UV illumination. For the reversibility studies, treated cells

were washed with PBS and incubated with fresh media for an additional hour before harvesting for plug preparation and gel analysis.

### EXAMPLE 9

#### Stimulation of In Vitro Cleavable-Complexes by Conjugated Inhibitors

5                   Compounds were evaluated as cleavable complex-forming inhibitors of calf thymus DNA topoisomerases I and II *in vitro*. The prototypical inhibitors etoposide (C-1) and camptothecin (C-2) were included as reference standards. Activities and specificities of compounds tested at ten micromolar are depicted in Figure 1A. The enzyme specificity and activity of  
10 three unconjugated inhibitors were consistent with measurements made using human enzymes and cellular assay systems as described in H.K. Wang et al., *Bioorg. & Med. Chem.* 2:1397 (1994) and Z.Q. Wang et al., *J. Med. Chem.* 33:2660 (1990). In contrast, compound 4a was less active than 1 at stimulating topoisomerase II-mediated DNA cleavage *in vitro* and not three fold more  
15 active based on an earlier study with a cell-based assay system reported in Z.Q. Wang et al., *J. Med. Chem.* 33:2660 (1990). Compound 4b has not been evaluated previously but it displayed similar properties as the *meta*-substituted congener. The conjugated inhibitors, S-1 and S-2, induced cleavable complexes with both types of enzyme however, their activities were reduced about two-  
20 fold relative to the unconjugated components 3, 4a and 3, 4b respectively. Results are reported in Figure 1A.

                  These results indicate that S-1 and S-2 displayed the combined selectivity of the unconjugated inhibitors but had lower relative activities at the single concentration tested. Compound S-2 was selected for further evaluation  
25 since the levels of S-2-induced and etoposide-induced DNA cleavage were similar. Concentration-dependent *in vitro* cleavage results using topoisomerase II as well as gel analysis of cleavage products are shown in Figure 1B. Etoposide (C-1), stimulated enzyme induced cleavage 1.5-, 3, and 12-fold at one, ten and fifty micromolar respectively. A different activity profile was  
30 displayed by compounds 4b and S-2 in that induced levels of cleavage were maximal at ten micromolar, being about 7- and 4-fold increased over enzyme

controls respectively. Compound **4b** was about four times more active than **C-1** at one micromolar and three-times less active at fifty micromolar. For similar treatments with compound **S-2**, only marginal stimulation of enzyme-mediated DNA cleavage was detected. Interestingly, some enzyme cleavage sites induced by compounds **4b** and etoposide were also distinguishable because at least two unique cleavage fragments were produced upon **4b**-treatment. Similar fragments were faintly visible as cleavage products induced by ten micromolar treatment with **S-2**. These results are reported in **Figure 1B**. Additional studies with topoisomerase I showed that the stimulation of cleavage activity by both **3** and **S-2** was not inhibited at higher test concentrations, however, the alkaline-treated reaction products were too numerous and heterogeneously-sized on autoradiographs to allow meaningful comparisons of cleavage site-specificities. Based on the overall findings, activity as cleavable complex-forming inhibitors was reduced by conjugation yet specific interactions in the presumed ternary complexes appeared to be unaffected.

#### EXAMPLE 10

##### Cell Growth Inhibition by a Conjugated Inhibitor

Compounds **3**, **4b** and **S-2** were evaluated as cell growth inhibitors of KB, KB-7d, KB-CPT100, and KB-VIN20c and  $IC_{50}$  values were established. The -7d cell line is a pleiotrophic multidrug resistant KB-subclone which exhibits 145-fold resistance to etoposide ( $IC_{50}$  for KB,  $0.16 \pm 0.04$  micromolar), in part because of reductions in both drug uptake and intracellular levels of topoisomerase II. Furthermore, KB-7d cells are cross-resistant to structurally diverse topoisomerase II-targeting drugs including doxorubicin, mAMSA and mitoxantrone but are not cross-resistant to 10-hydroxycamptothecin, a camptothecin analog described in P. Ferguson et al., *Cancer Res.* 48:5956 (1988). The -CPT100 cell line exhibits a 32-fold resistance to the growth-inhibitory effect of ( $IC_{50}$  for KB,  $9.8 \pm 3.2$  nM) and a reduced intercellular level, about two-fold, of topoisomerase I; these cells are not cross-resistant to **C-1**. The resistance phenotype of -CPT100 cells appears to be independent of topoisomerase I but the biochemical mechanism remains to be

fully characterized. Results from growth inhibition assays are given in Table 1.

Table 1  
Cell Growth Inhibition

Cell line	IC <sub>50</sub> (nM)		
	S-2	3	4b
KB	14 (10, 18)	7 (5, 9)	100 (72, 128)
KB-7d	15 (11, 19)	9 (5, 13)	388 (306, 460)
KB-CPT100	85 (71, 99)	575 (435, 705)	134 (112, 156)
KB-VIN20c	27 (21, 33)	18 (14, 22)	234 (144, 309)

Compound 4b was about 1.5-fold more active than C-1 at inhibiting KB cell growth with an estimated IC<sub>50</sub> of one micromolar and KB-7d cells were only about four-fold cross-resistant, in contrast to the cells marked resistance to etoposide. This type of improvement in activity against C-1-resistant cells has been reported for other C-4b-substituted epipodophyllotoxin derivatives in Y. Zhang and K.H. Lee, *Chin. Pharm. J.* 46:319 (1994). The relative growth inhibitory activities of 4b, like C-1, were similar for KB parent and -CPT100 cells and the KB-VIN20c sub-clone was about two-fold cross-resistant (Table 1). The unconjugated camptothecin analog, 3, inhibited KB cell growth with an estimated IC<sub>50</sub> value of 0.007 micromolar; it was equally active against the -7d subclone, and the -CPT100 cell lines was about 58-fold cross-resistant. The vincristine-resistant sub-line (-VIN20c) was marginally cross-resistant to 3, no more than 2-fold.

The relative inhibitory activities of compounds S-2 and 3 against the KB, -VIN20c and -7d cell lines were similar but the KB-CPT100 derivative was only six-fold cross-resistant to S-2. In fact, the conjugated inhibitor was marginally more active at inhibiting the growth of -CPT100 cells than compound 4b. Based on these findings, the growth inhibitory properties of compound S-2 closely resembles the behaviors of both the topoisomerase I- and topoisomerase II- inhibitory components.

**EXAMPLE 11**Cell Toxicity of a Conjugated Inhibitor

Compounds **3**, **4b**, and **S-2** were evaluated as cytotoxic agents and LD<sub>50</sub> values for a three-hour exposure were established. Results are reported in Table 2. Compound **4b** was equipotent at killing all KB cell lines. The conjugated inhibitor more closely resembled the topoisomerase II-inhibitory component in that KB, -CPT100 and -VIN20c cells were killed by **S-2** and **4b** at equimolar doses. However, KB-7d cells appeared cross-resistant to the cytotoxic action of the conjugate, which more closely resembled the behavior of **3**.

Table 2

Cytotoxicity Measured as Reduced Plating Efficiency

Cell Line	IC <sub>50</sub> (μM)		
	<b>S-2</b>	<b>3</b>	<b>4b</b>
KB	4 (1, 7)	10 (9, 11)	3 (1.5, 4.5)
KB-7d	>15	>16	5 (3.5, 6.5)
KB-CPT100	2 (1.1, 2.9)		2 (0.2, 3.8)
KB-VIN20c	4.6 (2.6, 6.6)	12 (16, 18)	3 (1.5, 4.5)

**EXAMPLE 12**Stimulation of Intracellular Protein-DNA Complexes by a Conjugated Inhibitor

Intracellular activities of compounds **3**, **4b** and **S-2** were compared to co-treatments with unconjugated inhibitors **3** and **4b** using the potassium-SDS precipitation assay method. Etoposide (**C-1**) was included as the reference standard for **4b** and **S-2**. Concentration-dependent protein-DNA complex formation in KB cells is shown in Figures 2A and 2B. Analysis of co-treated cultures showed that **3** and **4b** gave additive effects at low test concentrations (0.5-2.5 micromolar, Figure 2A) but at higher concentrations, co-treatment effects were indistinguishable from levels of protein-DNA

complexes induced by 4b-treatment alone (Figure 2B). At high test concentrations (7.5 - 60 micromolar), the intracellular activity of the conjugated derivative, S-2, was different from either 3 or 4b alone and in combination but was quite similar to the profile for C-1 (See, Figure 2B). However, at concentrations of one micromolar and lower, the intracellular activity of S-2 and 4b were indistinguishable from other but different from C-1, which elicited no measurable response (See, Figure 2A). Based on these comparisons, the relative intracellular activities of C-1, 4b and S-2 correlated well with the *in vitro* measurements except for one noticeable difference: the activities of 4b and S-2 in cells were clearly not dose-inhibitory at high concentration.

Further results show that S-2 was as effective as etoposide (C-1) in cleavage assays but was about ten-fold more active at inhibiting cell growth. To further characterize intracellular activities, compounds C-1, 3, 4b and S-6 were compared using a gel lysis assay method. Since this type of assay system is only sensitive enough to detect widespread double-stranded genomic breaks, the effect of topoisomerase II inhibitors like etoposide (C-1) will be readily observed whereas topoisomerase I inhibitors like camptothecin (C-2) will be marginally effective. Furthermore, genomic DNA integrity will generally be rapidly restored in cells upon drug-removal if the causative lesions involve topoisomerases II- cleavable complexes. All compounds were evaluated at fifty-times the IC<sub>50</sub> concentration for KB cell growth inhibition and for 3 and S-2, additional treatments at twenty-fold higher concentrations were examined. Results from a representative experiment are shown in Figure 4. Control DNA's were intact and remained in the agarose plug at the gel origin (lanes 1 and 2). High molecular weight DNA fragments were detected after treatment with eight micromolar etoposide (lane 3) and the integrity of the DNA was restored intracellularly upon drug removal (lane 4), consistent with the known properties of etoposide-induced topoisomerase II cleavable-complexes. Resolution of similar DNA species using PFGE has shown the induced fragments to be 50 kbp and, under some circumstances 300 kbp. Fragmentation was also detected following treatment with four micromolar 4b

(lane 5) and the lesions were reversible (lane 6), again consistent with the compounds known pharmacological action.

5 The camptothecin derivative, 3, was inactive at 0.4 micromolar (lane 10). At sixteen micromolar, marginal fragmentation was detected (compare lane 11 to the control in lane 1) which was not affected by reversing treatment (compare lane 12 to the control in lane 2). These effects of 3 are consistent with known actions of cleavable complex-forming topoisomerase I inhibitors. By comparison, marginal DNA fragmentation was detected for cells treated with the conjugated inhibitor S-2 at 0.8 micromolar (compare lanes 7 and 1) but at a twenty-fold elevated concentration, high molecular mass genomic fragments were detected (lane 8) and the integrity of the DNA was only partially restored following treatment reversal (compare lanes 8, 9 and 2). Based on these findings, intermediate levels of protein-DNA complexes were induced by compound S-2 relative to co-treatments with unconjugated components. At high concentrations, the majority of intracellular complexes induced by S-2 behaved as topoisomerase II-mediated DNA lesions.

20 The combined results of the experiments demonstrate that the topoisomerase I/topoisomerase II inhibitors of the present invention displayed lower activity *in vitro* relative to their unconjugated constituents, about two-fold at ten micromolar, but they retained target recognition specificity based on limited analysis of the activity profiles and induced cleavage products. Previous evaluation of semi-synthetic 4'-O-demethylepipodophyllotoxins with modifications at the C-4 $\beta$ - position revealed that bulky substituents are well tolerated. This same region corresponds to the "variable-substituent" domain in the composite pharmacophore model for topoisomerase II inhibitors proposed by MacDonald et al., *DNA Topoisomerase in Cancer* (Eds. Potmesil M, Kohn KW), pp 119-214, Oxford University Press, New York, (1991). It is also known that camptothecin analogs can bear C7-(aminoacylhydrazone)-formyl substituents without markedly affecting the ability to induce cleavable complexes. See, H.K. Wang et al., *Bioorg. & Med. Chem.* 2:1397 (1994).

30 In view of the current finding that conjugates bearing a substituent of substantial bulk also retained activity as topoisomerase I

inhibitors, this suggests the interaction domain(s) within the presumed ternary complex is not proximal to the C7-position in camptothecin.

Evaluation of compounds **4b** and **S-2** as topoisomerase II inhibitors revealed that the stimulation of *in vitro* cleavable-complexes was dose-inhibitory for **4b** and **S-2** but not for etoposide, which is known to act saturably. Self-suppressive effects seen with cleavable complex-forming inhibitors, ascribed to unfavorable topoisomerase-drug-template interactions, have been reported for DNA intercalators acting both *in vitro* and in cellular assay systems. See, K. Tewey et al., *Science (Wash. DC)* 226:466 (1984) and A. Bodley et al., *Cancer Res.* 49:5969 (1989). As the induction of intracellular protein-associated DNA complexes by **4b** and **S-2** was clearly not dose inhibitory, the variant behavior observed seen *in vitro* is believed to be dependent upon enzyme and/or template interaction(s) which are peculiar to the *in vitro* assay conditions.

The experimental support presented for the dual target specificity of compound **S-2** was based in part on measurements of cleavable complexes induced *in vitro* and in treated cells. Activity of the conjugated inhibitor relative to reference treatments was similar in the two assay systems and stimulatory effects of **S-2** on cellular protein-DNA complexes were distinguishable from the response to co-treatment controls. These observations suggest that the conjugate remained intact under cell culture conditions, which is consistent with the reported stability of the imine linkage. Additional indirect support for intracellular topoisomerase I and II-targeting by **S-2** came from growth inhibition and cytotoxicity studies, as a correlation was noted between these results and *in vitro* cleavage assay data when compounds **S-2**, **3** and **4b** were compared.



**EXAMPLE 13****In Vivo Effects of Compounds in Nude Mice  
with KB Cell Tumor Development**

5        Three to six week old NCr nude mice (Taconic immunodeficient  
mice and rats) were inoculated subcutaneously in each flank with  $2.5 \times 10^6$  cells,  
and tumors were allowed to grow. Treatment was started when the tumors  
were 250-500 mg as determined by caliper measurement and calculated  
according to the formula: tumor weight (mg) = (length (mm) x width (mm))/2.  
10        Drugs were given at the indicated doses on day 0 through 4, and tumor sizes  
were measured every several days. The tumor growth curves were generated  
as described previously. Toxicity was evaluated by changes in body weight.

      The results obtained in tests of compound S-1 are reported in  
Figure 4A. The results obtained in tests of compound S-2 are reported in  
Figure 4B.

15        The foregoing is illustrative of the present invention and is not to  
be construed as limiting thereof. The invention is defined by the following  
claims, with equivalents of the claims to be included therein.

**THAT WHICH IS CLAIMED IS:**

1. A compound useful as a topoisomerase I and topoisomerase II inhibitor, said compound having a structure according to formula I:



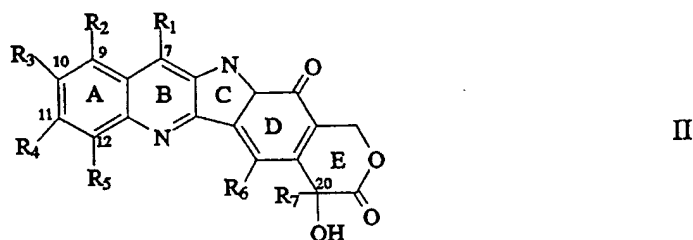
wherein:

- 5                     $T_I$  is a topoisomerase I inhibitor;  
                       $T_{II}$  is a topoisomerase II inhibitor; and  
                      L is a linking group.
2. A compound according to claim 1, wherein either  $T_I$  or  $T_{II}$  is both a topoisomerase I and a topoisomerase II inhibitor.
3. A compound according to claim 1, wherein  $T_I$  is a selective topoisomerase I inhibitor.
4. A compound according to claim 1, wherein  $T_{II}$  is a selective topoisomerase II inhibitor.
5. A compound according to claim 1, wherein  $T_I$  is a camptothecin group.
6. A compound according to claim 1, wherein  $T_{II}$  is an epipodophyllotoxin group.
7. A compound according to claim 1, wherein  $T_{II}$  is an amsacrine group.
8. A compound according claim 1, wherein  $T_{II}$  is an ellipticine group.

9. A compound according to claim 1, wherein  $T_{II}$  is an anthracycline antibiotic group.
10. A compound according to claim 1, wherein  $T_{II}$  is a mitroxantrone group.
11. A compound according to claim 1 in a physiologically acceptable carrier.

12. A method of inhibiting topoisomerase I and topoisomerase II, said method comprising contacting topoisomerase I and topoisomerase II *in vitro* or *in vivo* with an inhibitory effective amount of a compound according to claim 1.

13. A method according to claim 12, said compound having a structure according to formula II:



wherein:

- 5  $R_1$  is selected from the group consisting of H, alkyl, alkoxy, alkylaryl, hydroxyalkyl, haloalkyl, aminoalkyl, dialkylamino, dialkylaminoalkyl, cycloaminoalkyl, aryl, aryloxy, C-glycal,  $CO_2R_7$ , nitro, cyano, halo,  $SR_8$ ,  $NR_8R_8$ , O-glycosyl and  $L-T_{II}$ ; and
- 10  $R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are each independently H, amino, hydroxy, alkyl, alkoxy, alkylthiol, alkylamino, aminoalkyl, di(alkyl)amino, cycloaminoalkyl, aminoalkoxy, aryl, aryloxy, C-glycal, cyano, methylenedioxy, formyl, nitro, halo, azido, amido, hydrazino, any of the twenty standard amino acids bonded to the A ring via the amino-nitrogen atom,  $SR_8$ ,  $NR_8R_8$ , or O-glycosyl,  $L-T_{II}$ ; or  $R_3$  and  $R_4$  together form a 5- or 6-member aromatic or
- 15 dioxolane ring; and wherein where methylenedioxy comprises  $R_2$  and  $R_3$ ,  $R_3$  and  $R_4$ , or  $R_4$  and  $R_5$  taken together;
- $R_7$  is ethyl; and
- each  $R_8$  is independently selected from the group consisting of H, alkyl, alkylaryl, hydroxyalkyl, aminoalkyl, acyl, or aryl;
- 20 subject to the proviso that one of  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  is a substituent of the formula  $-L-T_{II}$ , wherein
- $L$  is a covalent linking group; and

$T_{II}$  is a topoisomerase II inhibitor.

14. A method according to claim 13, wherein  $T_{II}$  is both a topoisomerase I and a topoisomerase II inhibitor.

15. A method according to claim 13, wherein  $T_{II}$  is a selective topoisomerase II inhibitor.

16. A method according to claim 13, wherein  $T_{II}$  is an epipodophyllotoxin group.

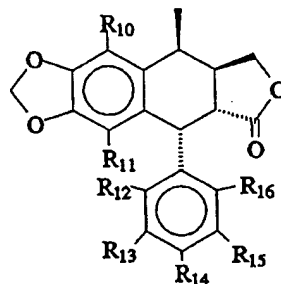
17. A method according to claim 13, wherein  $T_{II}$  is an amsacrine group.

18. A method according to claim 13, wherein  $T_{II}$  is an ellipticine group.

19. A method according to claim 13, wherein  $T_{II}$  is an anthracycline antibiotic group.

20. A method according to claim 13, wherein  $T_{II}$  is an mitoxantrone group.

21. A method according to claim 13, wherein  $T_{II}$  is an epipodophyllotoxin group of formula III-A:



III-A

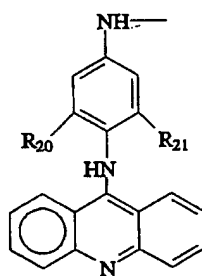
wherein:

$R_{10}$ ,  $R_{11}$ ,  $R_{12}$ , and  $R_{16}$  are each independently selected from the group consisting of H and halo;

$R_{13}$  and  $R_{15}$  are each independently alkoxy; and

5  $R_{14}$  is selected from the group consisting of H, hydroxyl, alkyl, and phosphate salt.

22. A method according to claim 13, wherein  $T_{II}$  is an amsacrine group of formula III-B

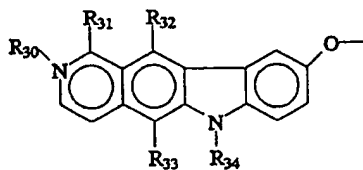


III-B

wherein

5  $R_{20}$  and  $R_{21}$  are each independently selected from the group consisting of alkoxy and alkylhydroxyl.

23. A method according to claim 13, wherein  $T_{II}$  is an ellipticine group of formula III-C



III-C

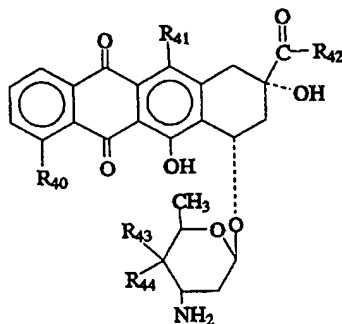
wherein

5  $R_{30}$  is H or aliphatic amine;

$R_{31}$  is H or  $-C(O)-N(R_{35})-(CH_2)_n-N(R_{36})(R_{37})$  wherein  $R_{35}$ ,  $R_{36}$ , and  $R_{37}$  are each independently selected from the group consisting of H and alkyl and n is an integer from 1 to 6;

$R_{32}$  and  $R_{34}$  are each indepently selected from the group consisting of H and alkyl; and  
 $R_{33}$  is alkyl.

24. A method according to claim 13, wherein  $T_{II}$  is an anthracycline antibiotic group of formula III-D



III-D

wherein

5

$R_{40}$  is H or alkoxy;

$R_{41}$  is hydroxyl or O—;

$R_{42}$  is alkyl, alkylhydroxyl, or  $\text{CH}_2\text{O}$ —; and

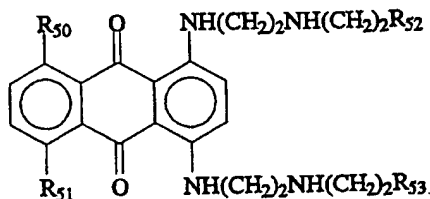
$R_{43}$  and  $R_{44}$  are each independently selected from the group

consisting of H and hydroxyl;

10

subject to the provisos that if  $R_{41}$  is hydroxyl, then  $R_{42}$  is  $\text{CH}_2\text{O}$ —; and if  $R_{42}$  is alkyl or alkylhydroxyl, then  $R_{41}$  is O—.

25. A method according to claim 13, wherein  $T_{II}$  is an mitoxantrone group of formula III-E

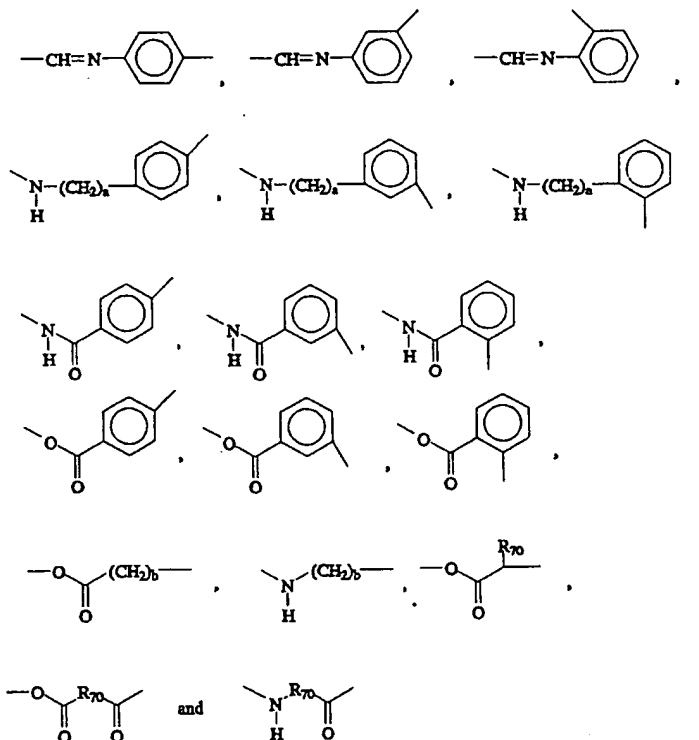


III-E

wherein

$R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  are each independently selected from the group consisting of OH or O—; subject to the proviso that one of  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  is O—.

26. A method according to claim 13, wherein L is a covalent linking group selected from the group consisting of:



wherein

a is a 0-3;

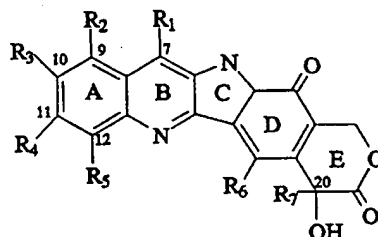
b is a 0-3; and

$\text{R}_{70}$  is selected from the group consisting of alkylene, alkenyl, and arylene.



27. A method of inducing cellular differentiation, said method comprising contacting a cancer cell with a differentiation effective amount of a compound according to claim 1.

28. A method according to claim 27, said compound having a structure according to formula II:



II

wherein:

5  $R_1$  is selected from the group consisting of H, alkyl, alkoxy, alkylaryl, hydroxyalkyl, haloalkyl, aminoalkyl, dialkylamino, dialkylaminoalkyl, cycloaminoalkyl, aryl, aryloxy, C-glycal,  $\text{CO}_2\text{R}_7$ , nitro, cyano, halo,  $\text{SR}_8$ ,  $\text{NR}_8\text{R}_8$ , O-glycosyl and  $\text{L-T}_{\text{II}}$ ; and

10  $R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are each independently H, amino, hydroxy, alkyl, alkoxy, alkylthiol, alkylamino, aminoalkyl, di(alkyl)amino, cycloaminoalkyl, aminoalkoxy, aryl, aryloxy, C-glycal, cyano, methylenedioxy, formyl, nitro, halo, azido, amido, hydrazino, any of the twenty standard amino acids bonded to the A ring via the amino-nitrogen atom,  $\text{SR}_8$ ,  $\text{NR}_8\text{R}_8$ , or O-glycosyl,  $\text{L-T}_{\text{II}}$ ; or  $R_3$  and  $R_4$  together form a 5- or 6-member aromatic or  
15 dioxolane ring; and wherein where methylenedioxy comprises  $R_2$  and  $R_3$ ,  $R_3$  and  $R_4$ , or  $R_4$  and  $R_5$  taken together;

$R_7$  is ethyl; and

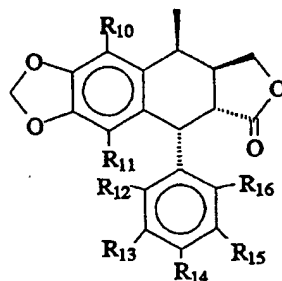
each  $R_8$  is independently selected from the group consisting of H, alkyl, alkylaryl, hydroxyalkyl, aminoalkyl, acyl, or aryl;

20 subject to the proviso that one of  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  is a substituent of the formula  $\text{-L-T}_{\text{II}}$ , wherein

L is a covalent linking group; and

$\text{T}_{\text{II}}$  is a topoisomerase II inhibitor.

29. A method according to claim 28, wherein  $T_{II}$  is both a topoisomerase I and a topoisomerase II inhibitor.
30. A method according to claim 28, wherein  $T_{II}$  is a selective topoisomerase II inhibitor.
31. A method according to claim 28, wherein  $T_{II}$  is an epipodophyllotoxin group.
32. A method according to claim 28, wherein  $T_{II}$  is an amsacrine group.
33. A method according to claim 28, wherein  $T_{II}$  is an ellipticine group.
34. A method according to claim 28, wherein  $T_{II}$  is an anthracycline antibiotic group.
35. A method according to claim 28, wherein  $T_{II}$  is an mitoxantrone group.
36. A method according to claim 28, wherein  $T_{II}$  is an epipodophyllotoxin group of formula III-A:



III-A

wherein:

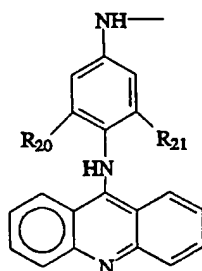
$R_{10}$ ,  $R_{11}$ ,  $R_{12}$ , and  $R_{16}$  are each independently selected from the group consisting of H and halo;

$R_{13}$  and  $R_{15}$  are each independently alkoxy; and

$R_{14}$  is selected from the group consisting of H, hydroxyl, alkyl,

5 and phosphate salt.

37. A method according to claim 28, wherein  $T_{II}$  is an amsacrine group of formula III-B

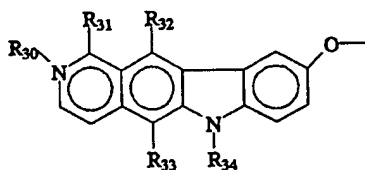


III-B

wherein

5  $R_{20}$  and  $R_{21}$  are each independently selected from the group consisting of alkoxy and alkylhydroxyl.

38. A method according to claim 28, wherein  $T_{II}$  is an ellipticine group of formula III-C



III-C

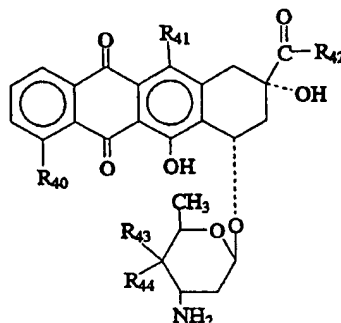
wherein

5  $R_{30}$  is H or aliphatic amine;

$R_{31}$  is H or  $-C(O)-N(R_{35})-(CH_2)_n-N(R_{36})(R_{37})$  wherein  $R_{35}$ ,  $R_{36}$ , and  $R_{37}$  are each independently selected from the group consisting of H and alkyl and  $n$  is an integer from 1 to 6;

$R_{32}$  and  $R_{34}$  are each independently selected from the group consisting of H and alkyl; and  
 $R_{33}$  is alkyl.

39. A method according to claim 28, wherein  $T_{II}$  is an anthracycline antibiotic group of formula III-D

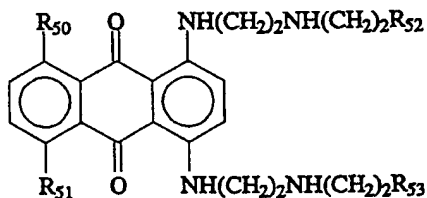


III-D

wherein

- 5  $R_{40}$  is H or alkoxy;  
 $R_{41}$  is hydroxyl or O—;  
 $R_{42}$  is alkyl, alkylhydroxyl, or  $\text{CH}_2\text{O}$ —; and  
 $R_{43}$  and  $R_{44}$  are each independently selected from the group consisting of H and hydroxyl;  
 10 subject to the provisos that if  $R_{41}$  is hydroxyl, then  $R_{42}$  is  $\text{CH}_2\text{O}$ —; and if  $R_{42}$  is alkyl or alkylhydroxyl, then  $R_{41}$  is O—.

40. A method according to claim 28, wherein  $T_{II}$  is an mitoxantrone group of formula III-E

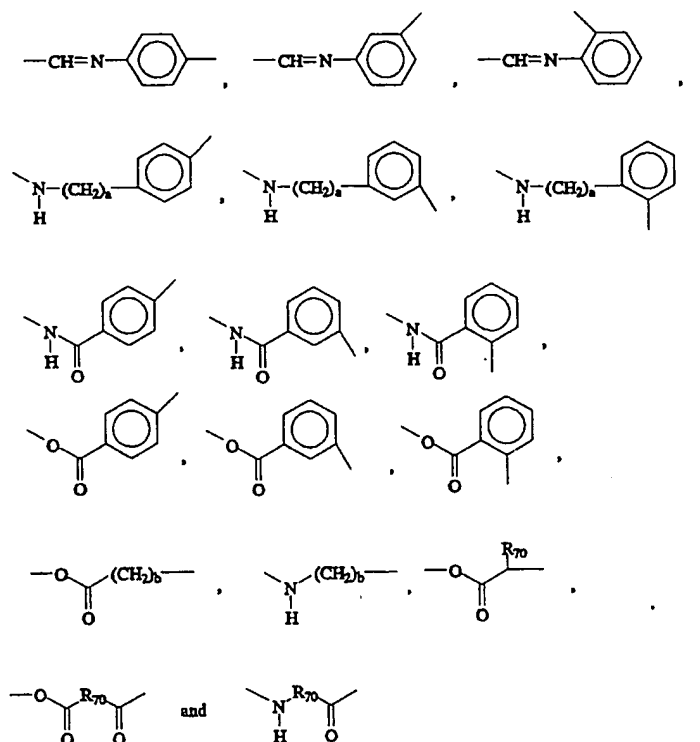


III-E

wherein

$R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  are each independently selected from the group consisting of OH or O—; subject to the proviso that one of  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  is O—.

41. A method according to claim 28, wherein L is a covalent linking group selected from the group consisting of:



wherein

a is a 0-3;

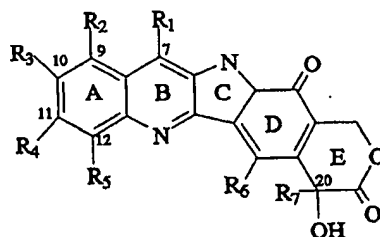
b is a 0-3; and

$\text{R}_{70}$  is selected from the group consisting of alkylene, alkenyl, and arylene.

42. A method of treating cancer in a subject in need thereof, said method comprising administering to said subject in an effective cancer treating amount a compound according to claim 1.

43. A method according to claim 42, wherein said cancer is selected from the group consisting of small cell lung cancer, testicular cancer, lymphoma, leukemia, esophageal cancer, stomach cancer, colon cancer, breast cancer, central nervous system cancer, liver cancer and prostate cancer.

44. A method according to claim 42, said compound having a structure according to formula II:



II

wherein:

5  $R_1$  is selected from the group consisting of H, alkyl, alkoxy, alkylaryl, hydroxyalkyl, haloalkyl, aminoalkyl, dialkylamino, dialkylaminoalkyl, cycloaminoalkyl, aryl, aryloxy, C-glycal,  $CO_2R_7$ , nitro, cyano, halo,  $SR_8$ ,  $NR_8R_8$ , O-glycosyl and  $L-T_{II}$ ; and

10  $R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are each independently H, amino, hydroxy, alkyl, alkoxy, alkylthiol, alkylamino, aminoalkyl, di(alkyl)amino, cycloaminoalkyl, aminoalkoxy, aryl, aryloxy, C-glycal, cyano, methylenedioxy, formyl, nitro, halo, azido, amido, hydrazino, any of the twenty standard amino acids bonded to the A ring via the amino-nitrogen atom,  $SR_8$ ,  $NR_8R_8$ , or O-glycosyl,  $L-T_{II}$ ; or  $R_3$  and  $R_4$  together form a 5- or 6-member aromatic or dioxolane ring; and wherein where methylenedioxy comprises  $R_2$  and  $R_3$ ,  $R_3$  and  $R_4$ , or  $R_4$  and  $R_5$  taken together;

15

$R_7$  is ethyl; and

each  $R_8$  is independently selected from the group consisting of H, alkyl, alkylaryl, hydroxyalkyl, aminoalkyl, acyl, or aryl;

subject to the proviso that one of  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  is a substituent of the formula  $-L-T_{II}$ , wherein

5

L is a covalent linking group; and

$T_{II}$  is a topoisomerase II inhibitor.

45. A method according to claim 44, wherein  $T_{II}$  is both a topoisomerase I and a topoisomerase II inhibitor.

46. A method according to claim 44, wherein  $T_{II}$  is a selective topoisomerase II inhibitor.

47. A method according to claim 44, wherein  $T_{II}$  is an epipodophyllotoxin group.

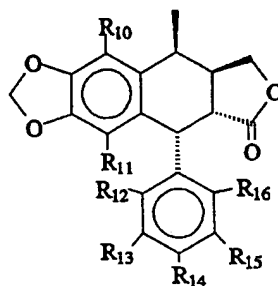
48. A method according to claim 44, wherein  $T_{II}$  is an amsacrine group.

49. A method according to claim 44, wherein  $T_{II}$  is an ellipticine group.

50. A method according to claim 44, wherein  $T_{II}$  is an anthracycline antibiotic group.

51. A method according to claim 44, wherein  $T_{II}$  is an mitoxantrone group.

52. A method according to claim 44, wherein  $T_{II}$  is an epipodophyllotoxin group of formula III-A:



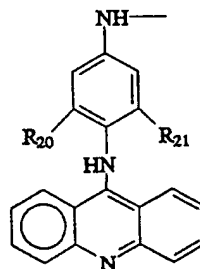
III-A

wherein:

5

$R_{10}$ ,  $R_{11}$ ,  $R_{12}$ , and  $R_{16}$  are each independently selected from the group consisting of H and halo;  
 $R_{13}$  and  $R_{15}$  are each independently alkoxy; and  
 $R_{14}$  is selected from the group consisting of H, hydroxyl, alkyl, and phosphate salt.

53. A method according to claim 44, wherein  $T_{II}$  is an amsacrine group of formula III-B



III-B

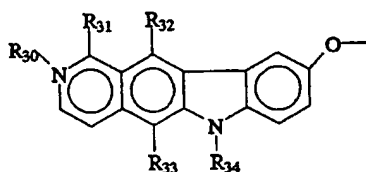
wherein

5

$R_{20}$  and  $R_{21}$  are each independently selected from the group consisting of alkoxy and alkylhydroxyl.



54. A method according to claim 44, wherein  $T_{II}$  is an ellipticine group of formula III-C

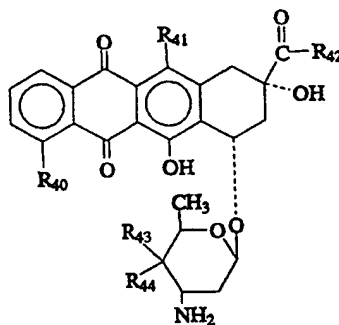


III-C

wherein

- 5  $R_{30}$  is H or aliphatic amine;  
 $R_{31}$  is H or  $-C(O)-N(R_{35})-(CH_2)_n-N(R_{36})(R_{37})$  wherein  $R_{35}$ ,  $R_{36}$ , and  $R_{37}$  are each independently selected from the group consisting of H and alkyl and n is an integer from 1 to 6;  
 $R_{32}$  and  $R_{34}$  are each independently selected from the group  
 10 consisting of H and alkyl; and  
 $R_{33}$  is alkyl.

55. A method according to claim 44, wherein  $T_{II}$  is an anthracycline antibiotic group of formula III-D



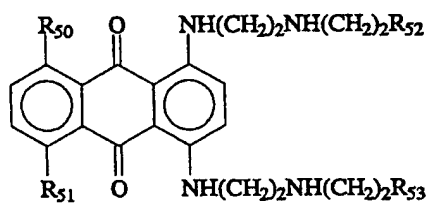
III-D

wherein

- 5  $R_{40}$  is H or alkoxy;  
 $R_{41}$  is hydroxyl or  $O-$ ;  
 $R_{42}$  is alkyl, alkylhydroxyl, or  $CH_2O-$ ; and  
 $R_{43}$  and  $R_{44}$  are each independently selected from the group  
 consisting of H and hydroxyl;

subject to the provisos that if  $R_{41}$  is hydroxyl, then  $R_{42}$  is  $\text{CH}_2\text{O—}$ ; and if  $R_{42}$  is alkyl or alkylhydroxyl, then  $R_{41}$  is  $\text{O—}$ .

56. A method according to claim 44, wherein  $T_{II}$  is an mitoxantrone group of formula III-E

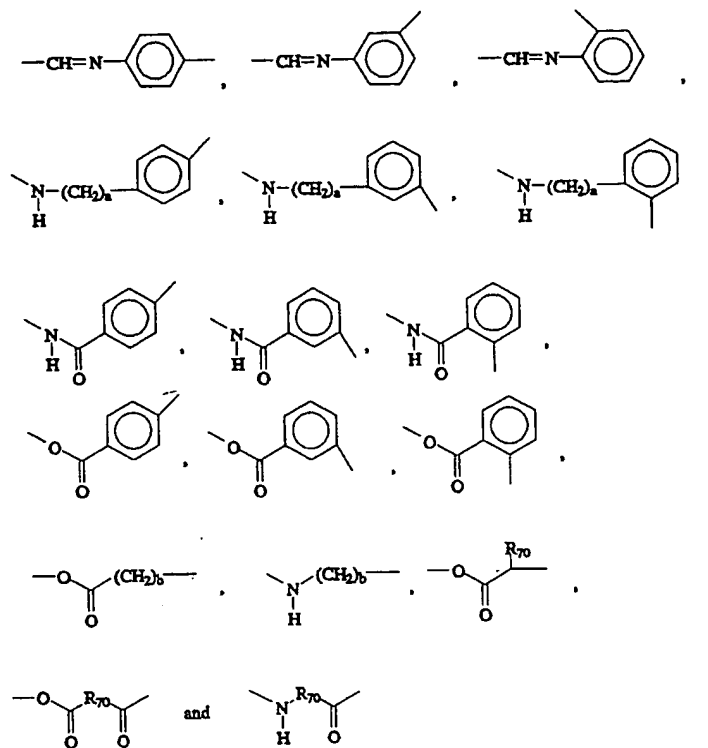


III-E

wherein

5  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  are each independently selected from the group consisting of OH or  $\text{O—}$ ; subject to the proviso that one of  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  is  $\text{O—}$ .

57. A method according to claim 44, wherein L is a covalent linking group selected from the group consisting of:



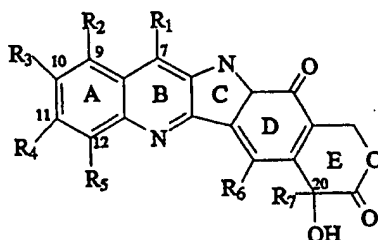
wherein

a is a 0-3;

b is a 0-3; and

$\text{R}_{70}$  is selected from the group consisting of alkylene, alkenyl, and arylene.

58. A compound useful as a topoisomerase I and topoisomerase II inhibitor, said compound having a structure according to formula II:



II

wherein:

- 5  $R_1$  is selected from the group consisting of H, alkyl, alkoxy, alkylaryl, hydroxyalkyl, haloalkyl, aminoalkyl, dialkylamino, dialkylaminoalkyl, cycloaminoalkyl, aryl, aryloxy, C-glycal,  $\text{CO}_2\text{R}_7$ , nitro, cyano, halo,  $\text{SR}_8$ ,  $\text{NR}_8\text{R}_8$ , O-glycosyl and  $\text{L-T}_{\text{II}}$ ; and
- 10  $R_2$ ,  $R_3$ ,  $R_4$  and  $R_5$  are each independently H, amino, hydroxy, alkyl, alkoxy, alkylthiol, alkylamino, aminoalkyl, di(alkyl)amino, cycloaminoalkyl, aminoalkoxy, aryl, aryloxy, C-glycal, cyano, methylenedioxy, formyl, nitro, halo, azido, amido, hydrazino, any of the twenty standard amino acids bonded to the A ring via the amino-nitrogen atom,  $\text{SR}_8$ ,  $\text{NR}_8\text{R}_8$ , or O-glycosyl,  $\text{L-T}_{\text{II}}$ ; or  $R_3$  and  $R_4$  together form a 5- or 6-member aromatic or
- 15 dioxolane ring; and wherein where methylenedioxy comprises  $R_2$  and  $R_3$ ,  $R_3$  and  $R_4$ , or  $R_4$  and  $R_5$  taken together;
- $R_7$  is ethyl; and
- each  $R_8$  is independently selected from the group consisting of H, alkyl, alkylaryl, hydroxyalkyl, aminoalkyl, acyl, or aryl;
- 20 subject to the proviso that one of  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ , and  $R_5$  is a substituent of the formula  $\text{-L-T}_{\text{II}}$ , wherein
- L is a covalent linking group; and
- $\text{T}_{\text{II}}$  is a topoisomerase II inhibitor.

59. A compound according to claim 58, wherein  $\text{T}_{\text{II}}$  is both a topoisomerase I and a topoisomerase II inhibitor.

60. A compound according to claim 58, wherein  $T_{II}$  is a selective topoisomerase II inhibitor.

61. A compound according to claim 58, wherein  $T_{II}$  is an epipodophyllotoxin group.

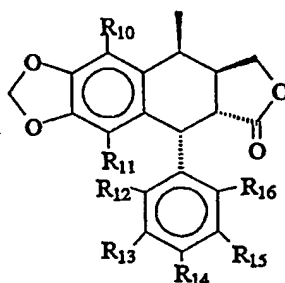
62. A compound according to claim 58, wherein  $T_{II}$  is an amsacrine group.

63. A compound according claim 58, wherein  $T_{II}$  is an ellipticine group.

64. A compound according to claim 58, wherein  $T_{II}$  is an anthracycline antibiotic group.

65. A compound according to claim 58, wherein  $T_{II}$  is an mitoxantrone group.

66. A compound according to claim 58, wherein  $T_{II}$  is an epipodophyllotoxin group of formula III-A:



III-A

wherein:

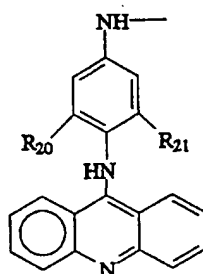
5

R<sub>10</sub>, R<sub>11</sub>, R<sub>12</sub>, and R<sub>16</sub> are each independently selected from the group consisting of H and halo;

R<sub>13</sub> and R<sub>15</sub> are each independently alkoxy; and

$R_{14}$  is selected from the group consisting of H, hydroxyl, alkyl, and phosphate salt.

67. A compound according to claim 58, wherein  $T_{II}$  is an amsacrine group of formula III-B

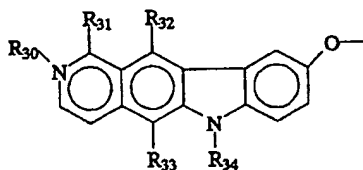


III-B

wherein

5  $R_{20}$  and  $R_{21}$  are each independently selected from the group consisting of alkoxy and alkylhydroxyl.

68. A compound according to claim 58, wherein  $T_{II}$  is an ellipticine group of formula III-C

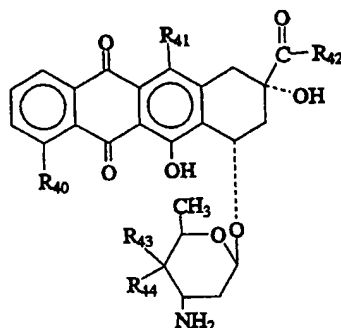


III-C

wherein

5  $R_{30}$  is H or aliphatic amine;  
 $R_{31}$  is H or  $-C(O)-N(R_{35})-(CH_2)_n-N(R_{36})(R_{37})$  wherein  $R_{35}$ ,  $R_{36}$ , and  $R_{37}$  are each independently selected from the group consisting of H and alkyl and  $n$  is an integer from 1 to 6;  
 $R_{32}$  and  $R_{34}$  are each independently selected from the group  
 10 consisting of H and alkyl; and  
 $R_{33}$  is alkyl.

69. A compound according to claim 58, wherein  $T_{II}$  is an anthracycline antibiotic group of formula III-D



III-D

wherein

5

$R_{40}$  is H or alkoxy;

$R_{41}$  is hydroxyl or O—;

$R_{42}$  is alkyl, alkylhydroxyl, or  $CH_2O$ —; and

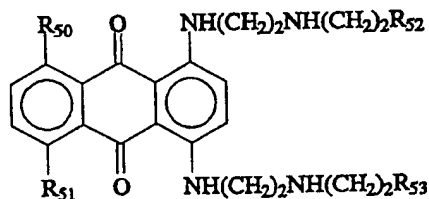
$R_{43}$  and  $R_{44}$  are each independently selected from the group

consisting of H and hydroxyl;

10

subject to the provisos that if  $R_{41}$  is hydroxyl, then  $R_{42}$  is  $CH_2O$ —; and if  $R_{42}$  is alkyl or alkylhydroxyl, then  $R_{41}$  is O—.

70. A compound according to claim 58, wherein  $T_{II}$  is an mitoxantrone group of formula III-E



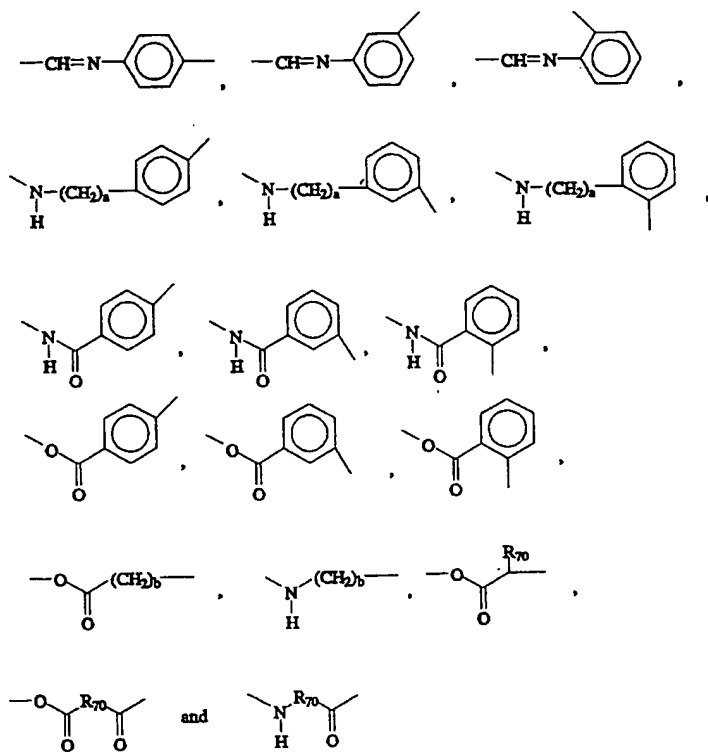
III-E

wherein

5

$R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  are each independently selected from the group consisting of OH or O—; subject to the proviso that one of  $R_{50}$ ,  $R_{51}$ ,  $R_{52}$  and  $R_{53}$  is O—.

71. A compound according to claim 58, wherein L is a covalent linking group selected from the group consisting of:



wherein

a is a 0-3;

b is a 0-3; and

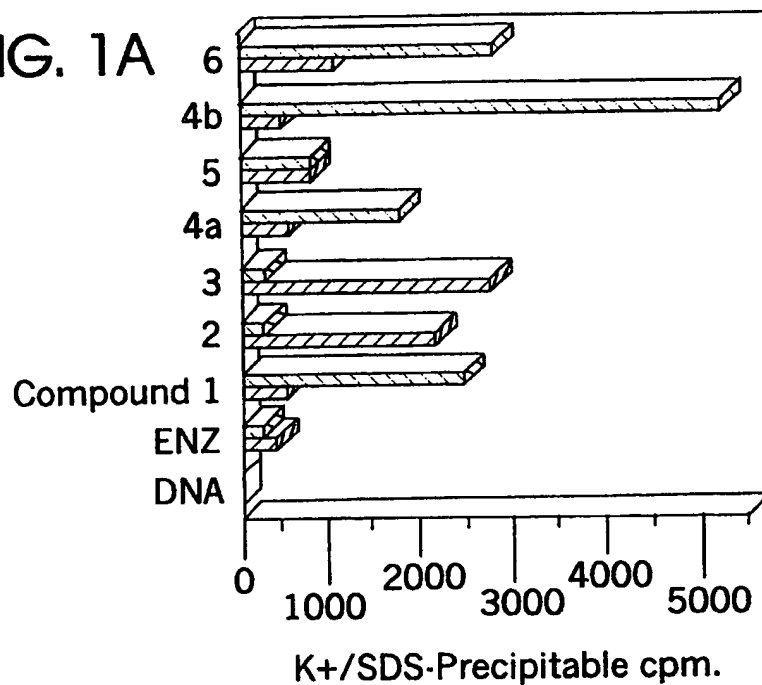
$\text{R}_{70}$  is selected from the group consisting of alkylene, alkenyl,

and arylene.



1/4

FIG. 1A



Compound 1 ( $\mu$ M)				4b			6			
IP	IX	1	18	58	1	18	58	1	18	58

FIG. 1B

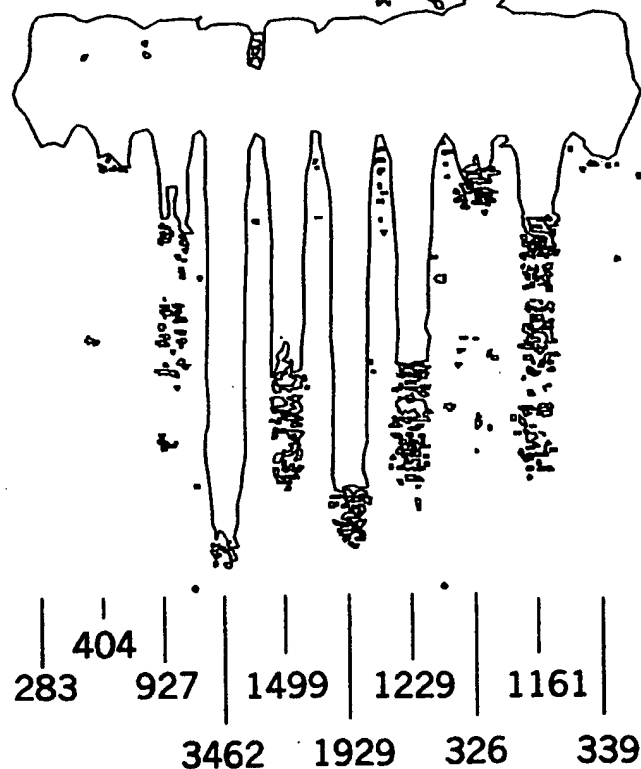


FIG. 2A

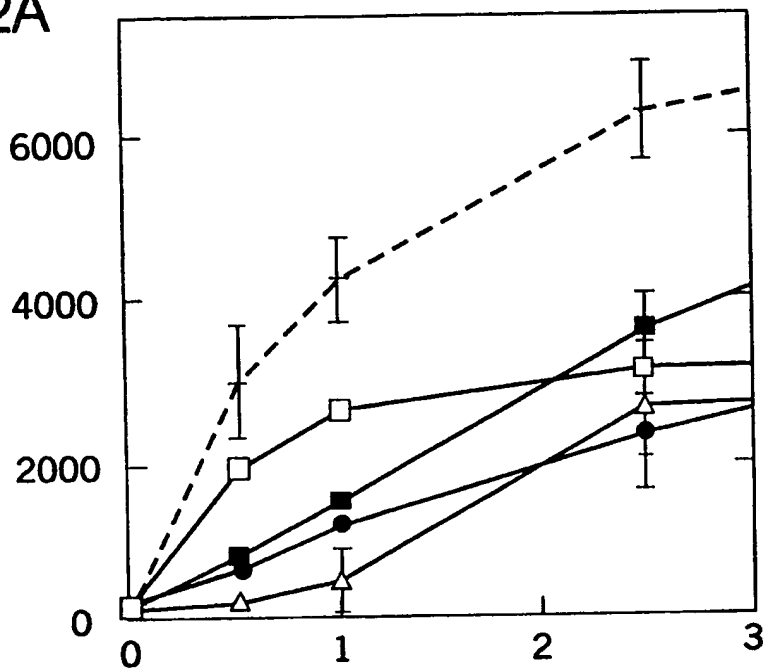


FIG. 2B

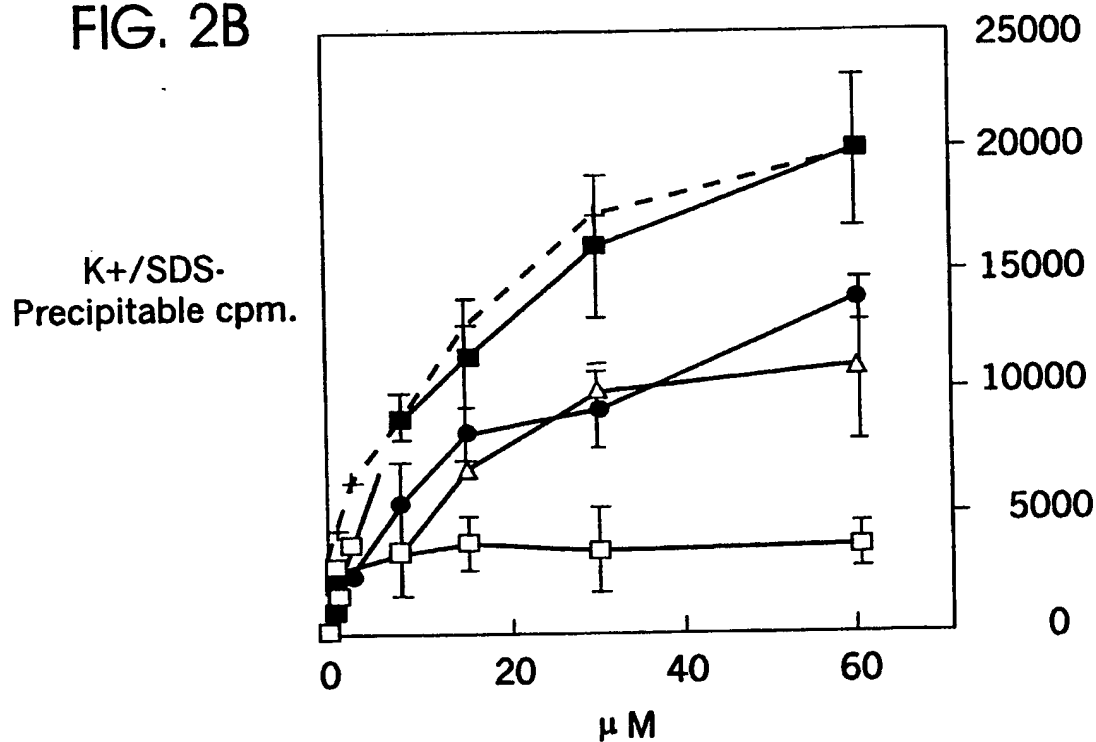


FIG. 3

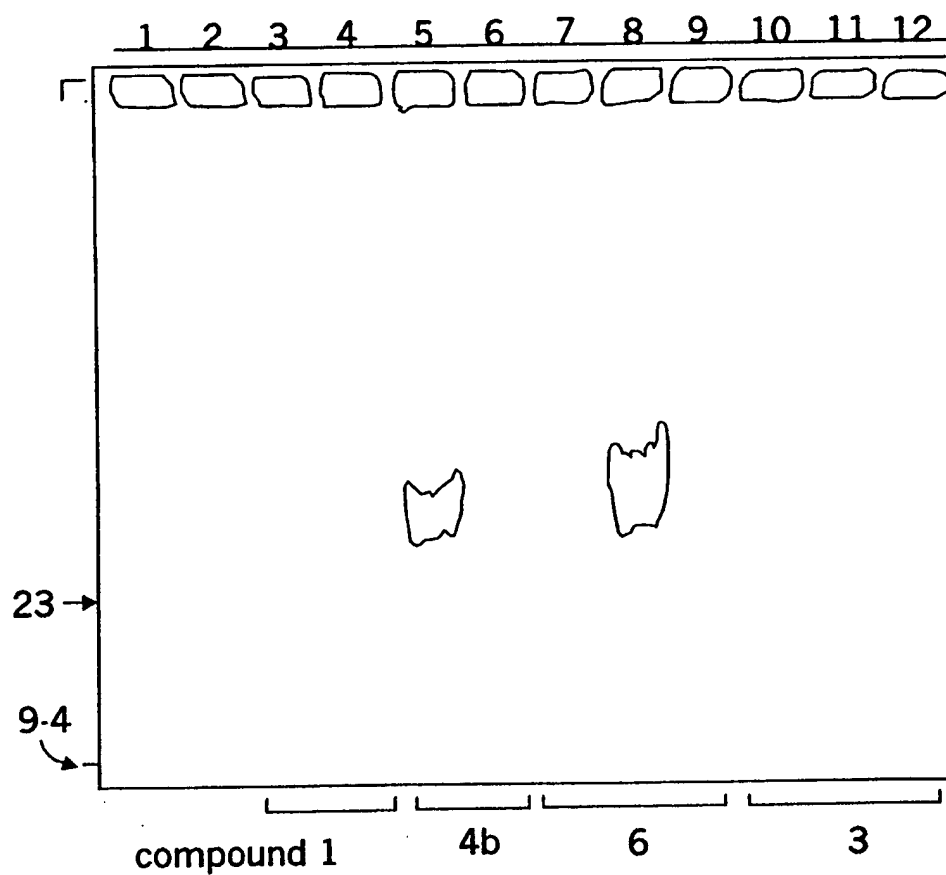


FIG. 4A

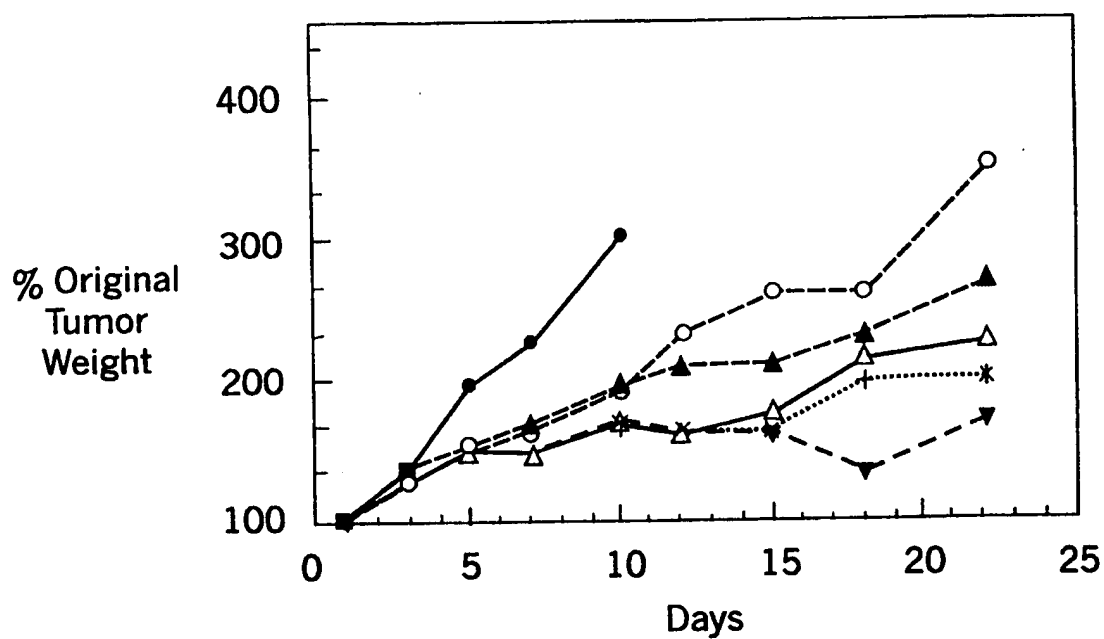
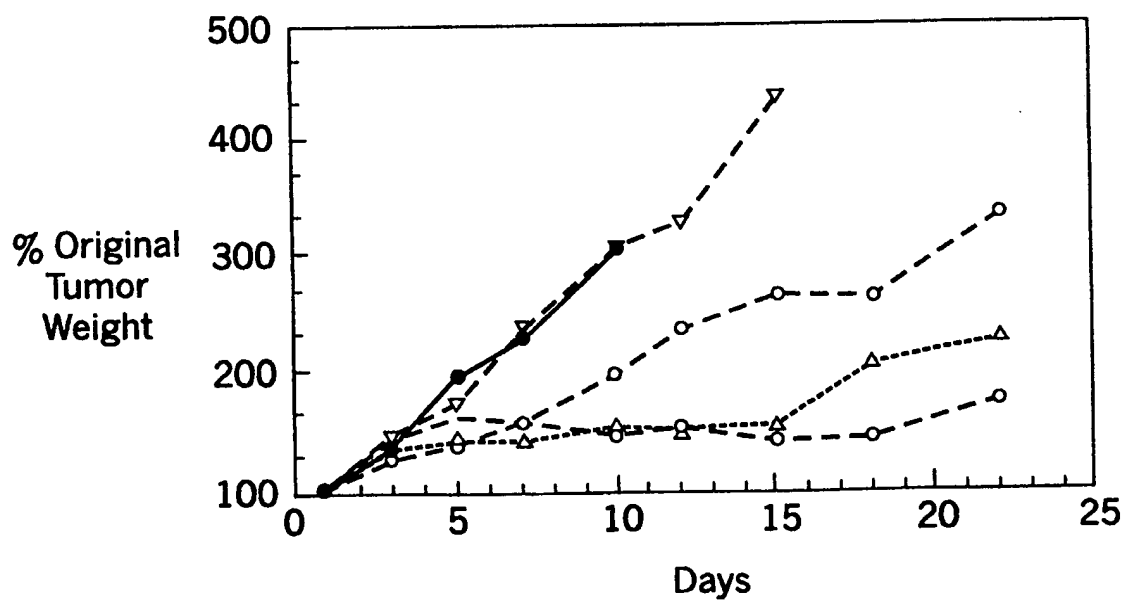


FIG. 4B



**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 47/48</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 98/40104</b> <b>(43) International Publication Date:</b> 17 September 1998 (17.09.98)
<b>(21) International Application Number:</b> PCT/US98/04852 <b>(22) International Filing Date:</b> 11 March 1998 (11.03.98)  <b>(30) Priority Data:</b> 08/815,890      12 March 1997 (12.03.97)      US  <b>(71) Applicants:</b> YALE UNIVERSITY [US/US]; Room 210, 155 Whitney Avenue, P.O. Box 208336, New Haven, CT 06520-8336 (US). THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL [US/US]; 308 Bynum Hall, CB #4105, Chapel Hill, NC 27599-4105 (US).  <b>(72) Inventors:</b> CHENG, Yung-Chi; 961 Baldwin Road, Woodbridge, CT 06525 (US). GUO, Xin; 7693 Palmilla Drive #2309, San Diego, CA 92122 (US). LEE, Kuo-Hsiung; 1426 Gray Bluff Trail, Chapel Hill, NC 27514 (US). BASTOW, Kenneth, F.; 1968 Lystra Road, Chapel Hill, NC 27514-8947 (US). WANG, Hui-Kang; 105 Fidelity Street, Carrboro, NC 27510-2062 (US).  <b>(74) Agents:</b> MAGRI, Karen, A. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US).	<b>(81) Designated States:</b> AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 11 February 1999 (11.02.99)	
<b>(54) Title:</b> COVALENT CONJUGATES OF TOPOISOMERASE I AND TOPOISOMERASE II INHIBITORS  <b>(57) Abstract</b>  The present invention provides covalent conjugates of topoisomerase I and topoisomerase II inhibitors. Such compounds have a structure according to the formula (I): $T_I-L-T_{II}$ wherein: $T_I$ is a topoisomerase I inhibitor such as a camptothecin group; $T_{II}$ is a topoisomerase II inhibitor such as an amsacrine, ellipticine, epipodophyllotoxin, or anthracycline antibiotic group; and L is a linking group. The compounds are useful for inhibiting topoisomerase I and II enzymes, for promoting cellular differentiation, and for treating cancer.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

# INTERNATIONAL SEARCH REPORT

International Application No

/US 98/04852

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>GUO X ET AL: "Novel biochemical and antitumor activity of the camptothecin-4beta-amino-4'-O-demethyl epipodophyllotoxin conjugates (Meeting abstract)."</p> <p>PROC ANNU MEET AM ASSOC CANCER RES;38:A4108 1997, XP002071384</p> <p>see abstract</p> <p>---</p> <p>-/--</p>	<p>1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

20 July 1998

Date of mailing of the international search report

27. 11. 98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3018

Authorized officer

Gonzalez Ramon, N

## INTERNATIONAL SEARCH REPORT

International Application No

: /US 98/04852

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 96 23794 A (ENZON INC) 8 August 1996</p> <p>see page 12 - page 15; claims 3,18,34; figures 2,3,6,7; examples 4,6,8-11,13 ---</p>	<p>1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71</p>
X	<p>DATABASE MEDLINE US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US AN=97459079, BASTOW KF ET AL: "Antitumor agents--CLXXIII. Synthesis and evaluation of camptothecin-4 beta-amino-4'-O-demethyl epipodophyllotoxin conjugates as inhibitors of mammalian DNA topoisomerases and as cytotoxic agents." XP002072004 see abstract &amp; BIOORG MED CHEM, AUG 1997, 5 (8) P1481-8, ENGLAND</p> <p>---</p>	<p>1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71</p>
Y	<p>EP 0 321 122 A (SMITHKLINE BECKMAN CORP) 21 June 1989</p> <p>see page 16, line 30-50; claims 1,13; table 8</p> <p>---</p>	<p>1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71</p>
Y	<p>WO 95 20567 A (UNIV KENTUCKY RES FOUND) 3 August 1995</p> <p>see page 11</p> <p>---</p> <p style="text-align: center;">-/--</p>	<p>1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71</p>



# INTERNATIONAL SEARCH REPORT

International Application No

/US 98/04852

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 332 811 A (LEE KUO-HSIUNG ET AL) 26 July 1994  see example 1; tables 1-3 ---	1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71
Y	PODDEVIN B ET AL: "Dual topoisomerase I and II inhibition by intoplicine (RP-60475), a new antitumor agent in early clinical trials." MOL PHARMACOL, OCT 1993, 44 (4) P767-74, XP002071386 UNITED STATES  see abstract see page 767, column 1 -----	1-6, 11-16, 21, 26-31, 36, 41-47, 52, 57-61, 66,71

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/04852

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 12-57  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☒ Claims Nos.: 1-4, 11, 12, 27, 42, 43  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:  
see INFORMATION SHEET PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See information sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Subject 1	Claims	6,16,21,31,36,47,52,61,66 part of 1-5,11-15,26-30,41-46 57-60,71
Subject 2	Claims	7,17,22,32,37,48,53,62,67, part of 1-5,11-15,26-30 41-46,57-60,71
Subject 3	Claims	8,18,23,33,38,49,54,63,68 part of 1-5,11-15,26-30 41-46,57-60,71
Subject 4	Claims	9,19,24,34,39,50,55,64,69 part of 1-15,11-15,26-30 41-46,57-60,71
Subject 5	Claims	10,20,25,35,40,51,56,65,70, part of 1-5,11-15,26-30 41-46,57-60,71

## Further defects under Article 17(2)(a):

In view of the large number of compounds, which are defined by the general definition in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application. (see Guidelines, Chapter III, paragraph 2.3).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

T/US 98/04852

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9623794	A	08-08-1996	US 5614549 A	25-03-1997
			AU 4913396 A	21-08-1996
			CA 2208841 A	08-08-1996
			EP 0807115 A	19-11-1997
-----				
EP 0321122	A	21-06-1989	AT 143368 T	15-10-1996
			AU 2639488 A	01-06-1989
			CA 1308102 A	29-09-1992
			CN 1034724 A,B	16-08-1989
			CN 1087637 A,B	08-06-1994
			CN 1083817 A,B	16-03-1994
			CY 2017 A	20-02-1998
			DE 3855575 D	31-10-1996
			DE 3855575 T	20-03-1997
			DK 663688 A	02-06-1989
			ES 2094721 T	01-02-1997
			FI 885569 A,B	02-06-1989
			GR 3021990 T	31-03-1997
			HK 81097 A	20-06-1997
			IE 74873 B	13-08-1997
			IL 88517 A	07-10-1994
			JP 1186893 A	26-07-1989
			JP 6033268 B	02-05-1994
			LU 90026 A	22-04-1997
			LU 90053 A	01-07-1997
			MX 9203744 A	01-09-1992
			PT 89111 A,B	29-12-1989
			US 5004758 A	02-04-1991
-----				
WO 9520567	A	03-08-1995	AU 6254594 A	15-08-1995
			CA 2182228 A	03-08-1995
			EP 0740650 A	06-11-1996
			JP 9509151 T	16-09-1997
-----				
US 5332811	A	26-07-1994	AU 632796 B	14-01-1993
			AU 5157190 A	26-09-1990
			EP 0461141 A	18-12-1991
			WO 9009788 A	07-09-1990
			US 5541223 A	30-07-1996
			US 5300500 A	05-04-1994
-----				